

Effect of MHA (methionine hydroxy analog) on
Protein Synthesis of Mixed Rumen Bacteria

By

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Abstract of Dissertation Presented to the Graduate Council
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EFFECT OF MHA (METHIONINE HYDROXY ANALOG) ON
PROTEIN SYNTHESIS OF MIXED RUMEN BACTERIA

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Incubations of mixed rumen bacteria, in vitro, were conducted to obtain information on the effect of methionine hydroxy analog (MHA) on rumen bacteria utilizing glucose, cellulose, or starch. The basal medium contained urea and 28 μ g of sulfur per ml in the form of magnesium sulfate.

In trials with glucose, MHA ranging from 0.04 (10 μ g S per ml) to 8 mg per ml of medium increased bacterial growth rate from two to three fold ($P < .01$) as measured by the synthesis of bacterial dry matter and crude protein, and the disappearance of glucose. The higher concentrations of MHA tended to be less effective.

The hydroxyl group, the double Ca salt, and the slight reducing potential of MHA were not essential for the stimulation of rumen bacterial growth. This was indicated in the

similarity of effects of MHA, DL-methionine, and the free acid form of MHA on rate of growth of rumen bacteria cultured with glucose.

The amino acid composition of rumen bacteria grown in glucose with or without MHA did not differ substantially. Nevertheless, there were some differences between our data and the literature in specific amino acid composition of rumen bacteria, suggesting that the populations developed in glucose differ from usual mixed rumen populations.

A comparison of the effects of adding equimolecular amounts of S in the form of several inorganic salts with that of MHA demonstrated that increased rumen bacterial growth was not due to the additional S provided by MHA.

Cysteine, cystine, and methionine were essentially similar to MHA in stimulation of rumen bacterial growth, but 15 other amino acids failed to increase growth rate.

Under accelerated rumen bacterial growth in the presence of MHA, the predominant catabolic product of glucose was lactic acid. MHA did not significantly increase the production of total VFA in a medium containing glucose.

The cellulolytic activity of bacteria that developed in a medium containing glucose, with or without MHA, was

found to be about half of that of rumen fluid from a steer fed hay.

MHA and methionine (10 μ g of S per ml) increased the growth rate ($P < .01$) of cellulolytic bacteria using purified cellulose as substrate. Cellulose digestion was from 5 to 15 per cent higher ($P < .01$) with MHA or methionine than with added sulfate or control at 24 hours. Proportional increases in bacterial N were observed. When incubation was extended to 48 hours, the MHA- and methionine-supplemented bacteria digested 10 to 20 per cent more ($P < .01$) cellulose than the control, but sulfate improved digestion almost as much as MHA.

Data on bacterial N suggest that MHA or methionine supplementation stimulated rate of bacterial growth more ($P < .01$) than sulfate at 24 hours. This rapid growth caused the logarithmic growth phase to end earlier. Cellular death and disintegration follow as indicated by lower bacterial N values at 48 hours than at 24 hours for the MHA- and methionine-supplemented bacteria.

Adding MHA or methionine to a medium with purified starch as the substrate doubled ($P < .01$) the amount of starch digested in 9 hours. This was due to faster growth of bacteria as measured by increased ($P < .01$) amount of bacterial N. At 18 hours of incubation, sodium sulfate

or methionine did not affect starch digestion, but with MHA a 5 per cent ($P < .01$) increase occurred. This increase in starch digestion was associated with an increase ($P < .05$) in bacterial N and ammonia consumption.

CHAPTER I

INTRODUCTION

Methionine-hydroxy-analog (MHA) when fed as a supplement to ruminants has been beneficial in only certain cases. The dietary response level may vary with breed, physiological activities, dietary protein, environmental conditions and ruminal microbe species composition. While any of these factors may be very important in causing the variable responses of ruminants to MHA supplementation, it should be worthwhile at the present time to investigate (1) the effects that MHA may have on bovine rumen bacteria incubated in vitro, (2) changes in rumen microbial populations, and (3) blood plasma lipoproteins of sheep fed MHA.

It has been frequently stated that an advantage in feeding MHA compared to methionine may be due to its slight solubility. This supposedly makes it unavailable for rumen bacteria and, therefore, equivalent to administration of methionine into the abomasum. Nevertheless, some observations indicate that microbes of the rumen are affected by MHA. For instance, it was reported by Patton et al. (1970a,

1970b) that protozoa numbers and ruminal lipid synthesis are increased by MHA. Salisbury et al. (1971) also showed that MHA is converted to catabolic products by rumen bacteria in vitro.

In vitro incubations were carried out to measure (1) the rate at which R. bacteria utilize glucose, cellulose, and starch; (2) the rate at which the bacterial populations grow by analyzing the bacterial N present at progressing times of incubation, and (3) the rate at which ammonia N is produced or consumed.

Since rumen bacteria cultured with glucose grew faster in the presence of MHA, a number of in vitro tests were made to help explain the nature of the stimulation as follows: (1) evaluation of functional groups of the MHA molecule associated with its activity, (2) amino acids composition of bacteria grown in presence of added MHA, (3) comparison of the effect of adding MHA with 18 different amino acids on growth and protein synthesis of rumen bacteria, (4) influence of other sources of sulfur (S) than MHA on the growth of rumen bacteria, (5) observations of the influence of MHA on the lag phase or logarithmic growth rate of rumen bacteria, and (6) comparison of VFA and lactic acid produced in cultures with and without MHA.

Observations were also made with sheep on the effect of dietary MHA on: (1) protozoa numbers and bacterial protein N in the rumen, and (2) amino acid composition of three ultracentrifugal fractions of lipoproteins in blood plasma.

CHAPTER II

LITERATURE REVIEW

It is generally believed that ruminants are not dependent on a dietary source of essential amino acids due to symbiosis with rumen microorganisms which provide an adequate source of protein for the host.

While rumen microbial protein production is beneficial in upgrading low quality proteins and utilizing nonprotein nitrogen, it may be an impeding factor in the utilization of very good quality protein. This has been shown by post-ruminal protein administration. Thus, all proteins may not be of equal value for the ruminant presumably due to variation in the balance of amino acids presented to the abomasum from different sources of protein.

Extensive studies on dietary sources and microbe synthesis of amino acids and proteins in ruminants were reviewed at the 10th Annual Ruminant Nutrition Conference (Purser, 1970).

A considerable amount of data obtained by Duncan et al. (1953), Purser and Buechler (1966), and Jacobson et al.

(1967) indicate that amino acid composition of rumen contents is not affected by the protein composition of the ration. The same finding was reported by Bergen et al. (1968), Liebholz (1969), and Meyer et al. (1967). However, some studies do not confirm this. Instead, they suggest that microbial protein quality is influenced by diet. Little et al. (1965) have demonstrated that ruminal bacterial protein from steers fed concentrate diets have higher biological value than from those fed hay.

Abdo et al. (1964) reported that rumen preparations from grazing animals were toxic to rats, while bacterial proteins from animals on dry feed were of good quality. The authors found evidence that sulfur containing amino acids in the mixed rumen protozoa and bacterial protein were inadequate for monogastric dietary requirements. It is apparent that amino acid nutrition of ruminants needs to be explored in depth. The present literature review will be limited mostly to the effects of methionine and MHA on ruminants. The following review has been organized under six subtitles which should aid in understanding the problems pertinent to this area of research.

Possibility of An Increase Rumen
Bacteria Production

Potter et al. (1968) fed soybean meal, urea, or both to wethers, and found no difference in ratios of amino acids in the abomasal contents. However, more protein nitrogen occurred in the abomasum of soybean meal fed animals.

Poley and Trenkle (1963) indicate that certain dietary proteins influence the amino acid composition of abomasal content and blood plasma. Leucine was high and lysine low in plasma and abomasal ingesta when sheep were fed corn gluten meal which is higher in leucine and low in lysine. But when urea or soybean meal were fed, the amino acid composition was similar.

Amos et al. (1970) reported a trend toward lower levels of total essential amino acids in the plasma of lambs fed increasing amounts of urea.

Little et al. (1968) demonstrated that most differences in plasma amino acids can be explained in terms of the quantities of protein reaching the abomasum when soybean meal is fed compared with urea. In their experiments 23 per cent less protein was observed in the abomasum of the urea group than those fed soybean meal.

Richardson and Tsien (1963) used fistulated identical twin steers and found that supplementation with soybean meal

resulted in the synthesis of a larger amount of all amino acids than when urea was fed.

Methionine or MHA Influence on
Nitrogen Retention When Added
to Urea Containing Rations

Klosterman et al. (1951a) reported improvements in N retention in sheep due to DL-methionine supplementation. The effect varied depending on the type of ration. Gallup et al. (1952) showed that three or six grams of methionine per animal per day increased N retention and digestion when fed to lambs with a 10.2 per cent protein ration containing urea, but failed when used with the same ration without urea.

In the study of Polan et al. (1970a) with lactating cows fed high urea rations, MHA not only improved N retention but also the digestibility of crude fiber and dry matter. When DL-methionine was substituted for an iso-nitrogenous amount of urea in a semipurified rations for lambs, Barth et al. (1959) and McLaren et al. (1965) showed that nitrogen retention was significantly increased. Similar results were obtained with L-tryptophan, but both amino acids combined did not further improve retention. No effect on organic matter or fiber digestibility was observed in the study by Barth et al. (1959).

Using a crossover experiment with wethers, Nimrick and associates (1970a) infused methionine, lysine, threonine, and urea, singly or in combination with each other. Methionine, but not lysine improved N retention. When fed together methionine and lysine increased N retention, but not as much as the combination of methionine, lysine, and threonine.

Nimrick et al. (1970b) tried the infusion of several amino acids into the abomasum of growing wethers fed a corn-soybean meal ration. The N retention values indicated that methionine is the first limiting amino acid. Other amino acids further improved N retention when combined with methionine. However, methionine was found not to be the first limiting amino acid in the extensive studies of Schelling and Hatfield (1967a, 1967b, 1968) on the effect of infusing casein or amino acids into the abomasum of growing lambs fed urea diets.

Brookes et al. (1969) failed to obtain an increase in N retention from oral administrations of DL-methionine, singly or in combination with other amino acids over that of lambs fed corn plus urea.

Influence of Methionine
on Digestibility

Gallup et al. (1952) obtained improved digestion in sheep when methionine was fed with higher protein rations, but not with lower protein rations. Klosterman et al. (1951b) found 0.2 per cent addition of DL-methionine by itself or with cobalt of no value on digestibility or in enhancement of the biological value of protein in sheep.

Bolsen and Woods (1970) used graded levels of methionine to obtain various N to S ratios and found no differences among their diets when measuring dry matter and protein digestibility, and nitrogen retention.

McLaren et al. (1965) reported no improvement of protein, organic matter, or fiber digestibility by lambs from adding DL-methionine, but they found increased N retention.

Working with lactating cows fed a high urea diet, Polan et al. (1970a) obtained large increases in dry matter and acid detergent fiber digestibility as the result of feeding 40 g of MHA per cow per day.

In contrast, Davis and Stallcup (1970) reported no significant effect on digestibility of sorghum silage. By feeding 4 g of DL-methionine daily to bulls, they also observed a decrease in digestibility of nitrogen-free extract and ether extract from feeding 44 g of MHA daily.

Johnson et al. (1970) studied the true digestibility of sulfur from different sources. They found that approximately 78 per cent S is digested from methionine and sodium sulfate, but only 36 per cent from elemental sulfur.

In vitro studies of Salisbury and Haenlein (1964) on the effect of 19 different amino acids on cellulose digestion indicated that only L-methionine had a stimulatory action.

Salisbury and Zikakis (1965) reported almost as much stimulation of cellulose digestion by rumen bacteria with MHA in vitro as that with methionine.

Trenkle et al. (1957) have also reported that methionine consistently improved the digestion of cellulose by rumen microorganisms in vitro.

Little et al. (1963a) found that addition of methionine to corn gluten meal did not improve in vitro cellulose digestion. But in a study by Bolsen and Woods (1970) methionine stimulated dry matter disappearance when added to ground or gelatinized corn.

Acord et al. (1966a, 1966b) studied the utilization of amino acids and other N sources for starch digestion in vitro. They observed that methionine, arginine, serine, valine, and glutamic acid had a slight stimulatory action; urea and ammonium salts gave the greatest response.

Methionine, as a Source of Sulfur for
Ruminants Fed Urea Rations

One reason for using methionine in early urea feeding trials was because of its sulfur content. Starks et al. (1954) fed purified diets containing urea that were low in sulfur to study the response of lambs to added elemental sulfur, sulfate, and methionine. All groups fed added S showed superior performance over the controls, but there were no statistical differences for weight gains between sulfur treatments or levels of supplementation, although more lambs finished the trial when fed methionine. The intermediate level of methionine 0.5 per cent produced the best gains compared to either a 0.2 per cent or a 0.7 per cent level.

Albert et al. (1956) evaluated the above three sources of S in rations similar to those used by Starks et al. (1954) and reported that rations with methionine (0.47, 0.64, and 0.82 per cent) gave the best results in the trial with the level 0.47 per cent giving the greatest gain. They concluded that about 70 per cent less sulfur was needed in the form of methionine than in the form of sulfate sulfur.

Garrigus et al. (1950) added 0.5 per cent S as methionine or 0.5 per cent elemental sulfur to a diet deficient in S--containing amino acids. Lambs receiving

methionine had greater gains ($P < 0.01$) than those fed unsupplemented or elemental sulfur diets; both supplementations were superior ($P < 0.01$) to the control in wool production.

Wool production was improved when the rations were supplemented with methionine in the three studies discussed above.

Trenkle et al. (1957) reported increased weight gains in lambs when methionine was fed in purified diets. However, the concentration of the amino acid fed was not given. Oltjen et al. (1960, 1962) obtained insignificant increases in rate of gain with sheep fed 0.4 per cent DL-methionine in similar purified diets.

Noble et al. (1955) studied the effect of adding 2 g of methionine per animal per day to practical rations for fattening lambs, one of which contained urea and the other soybean meal. The addition of methionine to the urea diet resulted in consistent increases in weight gains and feed efficiency. In two trials the urea diets with methionine were almost as good as those with soybean. Methionine added to the soybean meal ration had no observable effect.

Burroughs and Trenkle (1969) fed practical urea rations to lambs for 35 days. Some of the rations were supplemented with graded levels of MHA from 0 to 2.4 g per

day. Results were superior with MHA whether or not urea was present in the isonitrogenous rations. The lower levels of MHA (0.3, 0.6 g) gave the best results.

Mowat and Deelstra (1970) conducted two trials to study the effect that encapsulated methionine would have on utilization of proteins differing in solubility. In the first trial, methionine had no effect on efficiency of gain with soybean meal. With corn-urea and corn-blood-feather meal diets methionine increased gains by approximately 11 per cent and feed conversion by 9 per cent. In the second trial encapsulated methionine was fed at four levels in a corn-alfalfa-urea ration. No response was observed at the 0.2 per cent level, but the 0.4 per cent level increased gains by 12 per cent and feed efficiency by 10 per cent. The 0.6 per cent level resulted in much lower performance.

Hinds et al. (1961) reported no improvement from 0.5 per cent methionine, but a slight increase in gain and feed efficiency was observed when lysine and methionine were fed together with a high protein, pelleted ration offered ad lib to early weaned lambs.

Little et al. (1963b) found no significant increase in feed consumption and weight gain of lambs fed added lysine and methionine with corn gluten meal diets.

Beeson et al. (1960) and Gossett et al. (1962) fed cattle 10 g of methionine or MHA per head per day in one trial, and in another one fed 10 g each of methionine and lysine. They observed that methionine improved slightly a high-urea diet, but was inferior to lysine. The combination of the two amino acids was of no benefit with either a high-urea or an all protein ration. MHA was of no benefit at 5 g level and significantly depressed gain at the 10 g level.

Beeson et al. (1960, 1961) incorporated MHA in a high-urea supplement at levels of 5 or 10 g and fed 268 kg steers daily for 161 days. MHA had no beneficial effects at the 5 g level and proved to have adverse effects on rate of gain at the 10 g level.

Burroughs and Trenkle (1969) supplemented heifer calves weighing 209 kg initially with 3 g of MHA per head daily for 151 days. They obtained a 13 per cent higher rate of gain and a 10 per cent more efficient feed conversion. The high-urea MHA groups were also superior by the two criteria when compared to the soybean-urea (without MHA) group. The same workers reported later (1970) a trial with heavy steers weighing 336 kg initially in which levels of 3, 4.5, and 9.0 g of MHA per day were fed with a high-urea supplement ration for 133 days. Another treatment was 0.9 g of inorganic sulfur per head daily.

For the first 72 days the group fed 3 g of MHA per day gained 7 per cent faster and showed a 7 per cent higher feed conversion. The 4.5 g level improved performance over the control but not as much as the 3 g level. The 9 g level resulted in a negative response. The group receiving sulfur supplementation also responded significantly for the first 72 days. When data from the entire 133-day feeding trial were considered, no benefit was obtained from any level of MHA or the inorganic sulfur.

At the same time another group of lighter steers weighing 273 kg initially was fed a basal ration which was equivalent except that vegetable protein replaced the urea. No benefit was derived from either 1.5 or 3.0 g of MHA fed with this natural protein ration.

Burroughs et al. (1970) reported a study in which amino acids and proteins were compared as supplements for steers receiving a corn-urea ration. The results indicated that 3g of MHA per animal per day gave improvements of 4 per cent in gain and 5 per cent in feed conversion. These improvements were inferior to those obtained on addition of .45 kg of herring meal or soybean meal per animal per day. Using encapsulated methionine equivalent to 0.5 per cent N of the diet with seven-week-old early weaned dairy calves, Ingalls et al. (1970) found a slight increase in feed intake and weight gain with either urea or soybean diets.

Nelson et al. (1970) studied the effect of feeding 3 g of MHA per head of cattle per day under a variety of conditions. In the first trial which lasted for 148 days, MHA was not effective in improving weight gains of 300 kg yearlings fed a no-roughage, high-energy crude protein ration with some biuret. During the first month all groups receiving MHA had the best gains and one group continued to be superior for 84 days on the rations. Thereafter, higher performance was shown by the controls although the level of significance was not reported.

In a second trial with heifers averaging 306 kg body weight, those given MHA for 126 days performed significantly better with rations composed of corn silage with most of the nitrogen supplied from urea.

In a third trial lasting 112 days using rations low in energy and high in urea, MHA depressed performance. A fourth trial with steers and heifers calves weighing 175 kg and fed a high-protein ration, with and without 3 g of MHA for 225 days, showed slightly higher performance for the MHA group during the first two months. Throughout the remainder of the experimental period performance was very similar between the treatment groups.

Effect of Methionine on Lactation

Chandler (1970) calculated the net protein available from the diet to the cow at different levels of production. By applying certain assumptions and using average amino acid composition of bacterial protein he arrived at figures for net available amino acids. These values were then compared to values for the amino acids secreted in the milk proteins at various stages of production. In this way, he concluded that all amino acids are adequately available for a cow producing 10 kg of milk. Methionine and valine would become limiting at a level of 15 kg of milk, isoleucine at 20 kg, and others at increasing levels.

These calculations take into consideration only the protein secreted in the milk so that methionine, which is also metabolized for milk lipids, would likely become the first limiting amino acid for milk production.

Since rumen bacteria are known to synthesize S-containing amino acids from inorganic S, it is quite desirable to determine whether or not supplementation with methionine is necessary when the required S levels of the ration are met.

Jacobson et al. (1967) found that voluntary feed intake and milk production were significantly improved when a low S content ration for cows was supplemented with sulfur. In

this study neither free amino acids in plasma nor amino acid composition of hydrolyzed rumen microbes were significantly affected by S supplementation.

Jacobson et al. (1969) indicated that inorganic sulfur can only partially meet the sulfur requirement of the cow due to inefficient utilization of the supplemental sulfur.

Griel et al. (1968) first demonstrated that MHA increased milk production. This observation was made during a study of the use of MHA to prevent ketoses. MHA at levels of 0, 40, and 80 g per head per day was fed to several breeds of lactating cows. Forty grams were beneficial to all breeds. Eighty grams stimulated Holsteins and Jerseys but it was detrimental to Guernseys and Brown Swiss.

Bovine primary ketosis has been treated with MHA by McCarthy et al. (1968). In their study, ketosis symptoms were alleviated with either MHA or methionine. The lipid composition of blood serum and fatty acid composition of milk lipids returned to normal shortly after administration of the compounds.

Patton et al. (1970a) also found that total milk and milk fat production were increased by MHA supplementation. Chandler et al. (1970) reported similar increases in milk yield by intravenous injections of methionine or MHA.

Polan et al. (1970b) studied MHA levels from 0 to 0.8 per cent to determine the optimum level for lactation. MHA was added to a 14.5 per cent crude protein concentrate ration fed at 12 kg per day plus ad lib intake of corn silage containing 0.5 per cent urea. Peak milk production was obtained at an intake of 25 g of MHA per day. The fat content of the milk continued to rise with increasing levels of MHA. Feed intake was depressed at the highest level of MHA intake.

In a field trial involving 235 freshening dairy cows, Bishop (1970) fed 15 g of MHA per day for 10 days prepartum and 30 g postpartum. First-calf heifers fed MHA produced an average of 49.1 pounds of milk per day compared to 44.3 for unsupplemented heifers. Percentage fat content of the milk was not affected. The response of the older cows to MHA was related to age; they produced 4.2 pounds more milk and 0.33 pounds more fat than the total daily average when supplemented. The authors concluded that MHA promotes protein and lipid metabolism in older animals.

In contrast to the results discussed so far in this section, the literature has reports of ineffective supplementation with MHA or methionine. Burgos and Olson (1970) did not find significant differences in milk, fat, or solids-not-fat yield from cows receiving 40 g of encapsulated MHA per day.

Likewise, Martz et al. (1970) fed 12 g of encapsulated methionine per head per day for 120 days and found no significant differences in feed intake, milk yield, or milk composition.

Jacobson et al. (1970) used a double reversal design and demonstrated that total milk yield actually decreased an average of 2.4 kg per animal per day. Fat increased 0.5 per cent; therefore 4 per cent fat-corrected milk yield was unaffected as a result of including 80 g of MHA in the daily ration of high milk producing cows.

Data obtained by Chandler et al. (1970) indicate that intravenous infusions of methionine and MHA affect the balance of lipids with high milk producing cows by increasing endogenous lipogenesis. Blood lipids and milk production were significantly increased and serum methionine was slightly elevated.

Fisher (1969) used similar techniques, but failed to detect any rise in milk yield, feed intake, or a difference in milk composition. He infused intravenously 13 or 26 g of DL-methionine singly or in combination with lysine. Plasma levels of these amino acids were elevated.

Huntjens and Shultz (1970) in two trials used a 3x3 Latin square and observed that MHA had no significant effect on yield of milk, fat test for milk, or on the composition of circulating lipids and volatile fatty acids. The pH and volatile fatty acids in the rumen were unchanged. In this study, MHA was incorporated to a 3:1 and a 2:1 concentrate to roughage ratio at the rate of 1 g per 454 g of concentrate.

Rosser et al. (1971) studying the effect of MHA and whey components on milk fat production found that MHA corrected ($P < .01$) fat test depression in cows fed high concentrate rations.

Rumen Microbes Apparently Undergo Methionine Insufficiency

Methionine appears to affect not only the systemic protein and lipid metabolism of the ruminant, but also the microbiota of the rumen which make direct utilization of methionine and MHA.

Patton et al. (1968) noted changes in the microbial metabolism while studying the solubility of MHA and methionine in rumen fluid. The same investigators (1970a) observed that more total lipids were present in the rumen of animals given dietary MHA. They associated this synthetic activity with an increased concentration of protozoa.

Further data from the same laboratory (Patton et al. 1970b) indicated that the protozoa population became more abundant when a high corn ration was supplemented with MHA than when corn was fed alone. When hay was fed with a corn ration the largest protozoa numbers were obtained. A ration that contained corn, hay, and MHA was not evaluated, which would have been helpful in establishing whether MHA actually increased or simply prevented a decrease in protozoa numbers.

McCarthy et al. (1970) reported that in addition to a more dense protozoa population a change in dominant species also occurred when MHA was fed regularly. They postulated the possibility that rumen organisms may be experiencing an amino-acid insufficiency. These authors presented evidence of marked changes in the intraruminal metabolism. Normally dietary lipids are hydrolyzed by the microbes and then free unsaturated fatty acids hydrogenated, so that the host receives saturated long chain fatty acids. In the presence of MHA, microbes synthesize microbial complex lipids and these are stored in their protoplasts, lowering the free fatty acid content of the rumen fluid.

Salisbury et al. (1971) isolated and identified methionine sulfoxide, methanethiol, and CO_2 as catabolic products resulting from utilization of labeled methionine by rumen microorganisms. The radioactivity of the

trichloroacetic acid precipitated nitrogenous material which constituted a significant portion of the total activity was found to increase with incubation time.

Salisbury and Zikakis (1965) reported that DL-methionine was metabolized by rumen microorganisms during *in vitro* fermentations. Salisbury et al. (1971) demonstrated that MHA acted like methionine in preventing inhibition of cellulose digestion by methionine. The rumen microbes were observed to form the same catabolic products from MHA as from methionine.

The failure of ruminants to respond to MHA could be explained as an unnecessary supplementation due to any of the factors enumerated in the chapter on Introduction. The following discussion is an attempt to summarize and interpret the positive results reviewed above.

Overall the evidence suggest that the amino acid composition of proteins of mixed rumen bacteria populations is not affected by the type of ration fed to the host. Nevertheless, certain reports demonstrate that bacterial protein quality for the ruminant when measured *in vivo* may differ depending on the kind of ration. This may be due to a larger amount of bacterial protein which could provide greater amounts of all amino acids thereby raising the level of those which are present in lowest quantity to meet the animal's demand, at certain physiological stages.

The fact that MHA or methionine increases N retention when added to urea rations indicates that they either increase bacterial growth or become directly available to the host animal. The latter has been postulated in explanation for results of MHA feeding. However, studies that show increased digestibility of forages when MHA or methionine have been added to the ration point to faster bacterial growth as the preferred explanation.

Digestibility has also been shown to improve upon supplementation of urea rations with inorganic sulfur. This has led some to believe that the function of MHA or methionine is to supply necessary S to S deficient rations. However, when animal performance of growing cattle rather than digestibility is the criterion considered, inorganic S appears to be less effective than MHA or methionine in most studies.

Improved milk yield with MHA supplementation has been reported while methionine or sulfate supplementation has usually been unsuccessful. An important difference in the rations of lactating cows and growing cattle is the large proportion of concentrates in dairy rations suggesting that amylolytic bacteria may respond selectively to MHA. A few observations have been made suggesting that MHA or methionine feeding results in larger numbers of rumen protozoa, affects

bacterial metabolism, and causes catabolism by rumen microbes.

MHA affects rumen microbial metabolism in a way similar to that of methionine. It is possible to speculate that this similar action of the two metabolites is mediated through faster rumen bacterial growth. This is contrary to the commonly held view that MHA bypasses the rumen activity and affects the host directly.

CHAPTER III

METHODS AND MATERIALS

In Vitro Procedures

Bacterial Cultures and Media Composition

In vitro cultures of rumen bacteria in the absence of protozoa were carried out using a total volume of 160 ml of culture media in 250 ml bottles fitted with glass tubes permitting continuous CO₂ bubbling through the media. The temperature was maintained at 39 C thermostatically.

A volume of 100 ml of basal medium contained 92.5 ml of McDougall's artificial saliva (McDougall, 1948) and 7.5 ml of a solution containing (a) 25 mg valeric acid, (b) 50 mg para-amino benzoic acid (PABA), and (c) 20 µg biotin. One or 2 ml of a solution that contained 126 mg of urea per ml and 0.1 ml of a CaCl₂ solution (40 mg per ml) were added to each fermentation bottle. This medium was saturated with CO₂ and the pH adjusted to 6.9 with Na₂CO₃ prior to inoculation and after each sampling period. Different substances such as MHA, DL-methionine, or other amino acids, Na₂SO₄ or other S-containing salts, were added to the culture bottles, depending on the treatment.

In early trials, a sufficient quantity of MHA was added to saturate the basal media and the excess removed by filtration. This solution was found to contain 7.78 mg of MHA per ml (0.8 per cent). In later trials, the additives for each treatment were dissolved in 10 ml of water and 2 ml of this solution transferred to each incubation bottle; the controls received 2 ml of water. The amounts in mg dissolved per 10 ml were: MHA, 35.6; DL-methionine, 37.4; Na_2SO_4 , 35.5. Thus, each treatment received 10 μg of S per ml of total media or 1.6 mg of S in the 160 ml total solution in each bottle.

Either glucose, cellulose, or starch were added to the media in the dry form. In some cases glucose was added as an aqueous solution, and the insoluble substrates were put in suspension by adding 57 g of Solka Floc¹ or starch in 750 ml of basal media using a Waring blender. Aliquots containing approximately 3 g of substrate were taken during agitation and transferred to each bottle.

Inoculum

For the glucose and cellulose experiments, whole rumen content was obtained from a rumen fistulated steer fed coastal bermuda grass hay, ad lib and 1 kg of soybean meal plus trace

¹Solka Floc, Brown Company, 733 Third Avenue, New York, New York.

mineralized salt and defluorinated phosphate. Vitamins A, D, and E were also provided. Water was available continuously. The rumen inoculum for the starch experiments was obtained from a fistulated steer fed a ration consisting of three-fourths concentrate (soybean meal, corn meal 1:8), one-fourth hay, and trace mineralized salt, defluorinated phosphate and vitamins A, D and E.

Rumen content was withdrawn with a large plastic tube having a rubber plug on the bottom which could be opened and closed by use of a metal rod passing through the tube. Upon withdrawal from the rumen, the fluid was immediately strained through four layers of cheesecloth and centrifuged at 250 RCF for 10 minutes. In this manner the protozoa and feed particles were sedimented. Twenty ml of the supernatant fluid containing mixed rumen bacteria were added to 140 ml of basal medium in each fermentation bottle. The centrifuged rumen fluid (CRF) and the basal medium were mixed in a large container and saturated with CO_2 . Thereafter, the mixture was siphoned into pre-warmed fermentation bottles containing the CO_2 saturated substrate suspension or solution, together with the corresponding treatment substances.

At the end of each specified period, 20 ml aliquots of total medium were pipetted while the suspension was manually agitated to insure homogeneity. The aliquots were then transferred to previously weighed 50 ml, thick-walled centrifuge tubes.

Preparation of the Free Acid Form of
MHA (MHA-H) and Test of Identity

It was necessary to find a solvent more polar than water in which larger amounts of $(\text{MHA})_2\text{-Ca}$ could be ionized. The most adequate solvents were 2 per cent HCl in water and a mixture of 37 ml concentrated acetic acid, 10 ml of water, and 3 ml of concentrated HCl as recommended in the procedure for analysis of MHA. The second solvent was used because the solution in 2 per cent HCl starts to separate in brown microcrystals after 6 hours, but it is quite stable in acetic acid/HCl/H₂O.

Neutralization of these solutions of MHA with NaOH causes the material to precipitate out during a few hours of standing. When the acid solution of MHA is treated with an equimolecular amount of EDTA or oxalate to combine with the Ca present and then neutralized, the neutral solution was very stable. Oxalate was chosen for the final preparation because the Ca-oxalate formed can be separated by decantation or filtering, obtaining a pure solution of MHA-H. The white precipitate was recognized as $\text{CaC}_2\text{O}_4\cdot\text{H}_2\text{O}$ by calculating its solubility products in three solvents, and by running a melting point of the isolated material, or in mix with pure $\text{CaC}_2\text{O}_4\cdot\text{H}_2\text{O}$, MP = 200 C.

The possible hazard of oxidation of MHA exists when

dissolved in an acid medium. A possible site of oxidation is the -OH group to a ketone group. A more susceptible site of oxidation should be that of the sulfide to a sulfoxide group.

It was desirable to establish the molecular structure of the resulting substance and this was attempted with NMR spectroscopy.

To avoid spurious bands due to solvent interaction and to study the form of the molecule at neutral pH, MHA-H was transferred to water by slow evaporation of the water/acetic acid/HCl solvent aided by a N stream blown on a thin layer of solution. Dry MHA-H was redissolved in water. The MHA-H obtained in this manner was freely soluble in water.

Since a direct comparison of the spectra of $(\text{MHA})_2\text{-Ca}$ and MHA-H was not possible due to low solubility of the Ca salt in water, the spectrum of MHA-H was compared to the theoretical spectrum that would correspond to MHA-H. Such a model is as follows:

The proton nuclear magnetic resonance absorption splittings are designated by numbers 1, 2, 3, and 4 on the structural formula for MHA-H (Table 1). The NMR spectrum of the sulfoxide should differ only in the position of absorption of the 1 and 2 protons; they would be shifted

Table 1

Nuclear Magnetic Resonance Absorption Splittings
of MHA-H and Comparison with the Experimental Ratios

				OH C H-COO
	H ₃ C - S -	CH ₂ -	CH ₂ -	
	1	2	3	4
Splitting:	Singlet	Triplet	Multiplet	Triplet
		No. of Protons	Integral Values	Approximate Ratios
1 -CH ₃	Singlet	3	155-55=100	3
2 -CH ₂	Triplet	2	59	2
3 -CH ₂	Multiplet	2	55	2
4 -CH	Triplet	1	31	1

downfield 0.3 ppm in CCl_4 solution (Silverstein and Bassler, 1964).

A larger downfield shift would be expected in a polar solvent as water, i.e., for protons near a solvated site, such as 1 or 2, a minimum of 0.8 ppm downfield is to be expected for the methyl singlet. The splitting of each high resolution peak is also numbered accordingly. The number of protons is correlated with integrated areas experimentally obtained.

A small singlet appears at 4.25 ppm (Figure 1) or 0.9 ppm downfield from the main MHA-H singlet.

It represents approximately 15 per cent of the MHA-H methyl singlet and is probably due to no more than 15 per cent of the R-S-CH_3 being oxidized to $\text{R}-\overset{\text{O}}{\underset{\text{||}}{\text{S}}}-\text{CH}_3$.

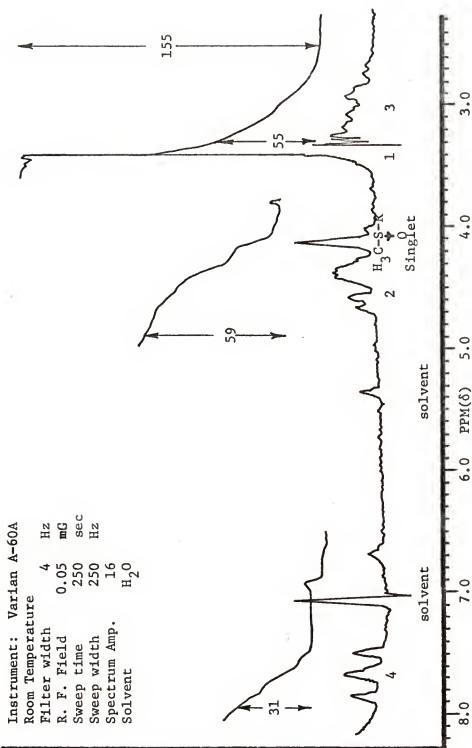


FIGURE 1. NMR SPECTRUM OF MHA-H

Dry Matter

To each of the 20 ml aliquots of medium, 5 ml of 10 per cent Na-tungstate and 5 ml of 1.07 N sulfuric acid were added as described by Shultz and Shultz (1970). After a minimum of 4 hours, the tubes were centrifuged at 2000 RCF for 20 minutes, following which the supernatant fluids were decanted and refrigerated for determinations of VFA, lactic acid, glucose, and ammonia depending on the particular experiment.

The above sediment was resuspended with distilled water, centrifuged and the supernatant washing discarded by gentle suction through a capillary tube with a water aspirator. The sediments in the centrifuge tubes were dried at 80 C.

The dry matter (DM) in the tubes was weighed to 0.1 mg with an analytical balance. DM weight was obtained by subtracting the empty tube weight. This tungstic acid insoluble DM is referred to as bacterial DM in the present study.

Bacterial Nitrogen

To determine the amount of total N present in the bacterial DM, the micro-Kjeldahl procedure was used (Kirk, 1950). Sulfuric acid-hydrogen peroxide oxidation was carried out

directly within the 50 ml tubes using 2 to 5 ml of concentrated H_2SO_4 depending on the amount of DM present. Small volumes of H_2O_2 were added at regular intervals, to the boiling sulfuric acid.

Two procedures were used to analyze for ammonia in the sulfuric acid digests. One method was a modified micro-Kjeldahl procedure utilizing the Aminco all glass apparatus. The digests were washed into appropriate flasks, made alkaline with 8 ml of 30 per cent NaOH and the liberated NH_3 collected in 5 ml of saturated boric acid. Methyl-red-methylene blue (1:1 v/v) indicator solution was added to the boric acid. The ammonia which combined with the boric acid was determined by titrating to the blue end point with 0.02 N HCL. Total mg of N in the DM of the 20 ml aliquot of total media was calculated by multiplying ml x 0.02 x 14.0067.

The second procedure used to analyze for N in the digests was the phenol-nitroprusside, alkaline-hypochlorite photolorimetric method (Kaplan, 1965). Two to 5 ml of sulfuric acid-peroxide digests were diluted to 30 ml with distilled water and 0.01 to 0.05 ml of this dilution mixed with 5 ml of phenol-nitroprusside solution and 5 ml of alkaline hypochlorite solution. The optical density (OD)

of the blue color which developed after 30 minutes was measured in the Beckman D.U.-Gilford spectrophotometer at 560 mμ. The mg of N present in the DM corresponding to 20 ml of total media was calculated by developing a standard curve ranging from 0 to 20 μg of ammonia N. The slope⁻¹ (μgN/OD) of the standard curve plot was determined and used as follows:

$$\text{mg N in DM} = \frac{\text{OD} \times \text{slope}^{-1} \times 30}{0.05 \times 1000} .$$

and referred to as bacterial N.

Ammonia N

Ammonia N was determined in the clear supernatant resulting from the 20 ml of total media and 10 ml of tungstic acid (TA) mix after centrifugation of bacterial cells. The phenol-nitroprusside method was used on 0.05 ml aliquot of supernatant. Standards for the calibration curve ranging from 0 to 20 μg were adjusted to the pH of the supernatants.

The mg of ammonia N in the 160 ml total volume of an incubation bottle were calculated as follows:

$$\text{mgN}/160 \text{ ml} = \frac{\text{OD} \times \text{slope}^{-1} \times 30 \times 160}{0.05 \times 20 \times 1000}.$$

Slope⁻¹ refers to the $\mu\text{g N}/\text{OD}$ from the standard curve.

Lactic Acid

Lactic acid was determined in the supernatants by the para-hydroxydiphenyl method of Barker and Summerson (1941). Deproteinization of solutions with ZnSO_4 was not necessary as they recommended since protein precipitation had been done with TA.

Elimination of soluble carbohydrates was done by CuSO_4 complexation, absorption of the complex on $\text{Ca}(\text{OH})_2$, and removal by centrifugation. Five-tenths or 0.1 ml of the CuSO_4 supernatant solutions were used for the color reaction. Blank and standard solutions were developed. Zero OD at 560 m μ wavelength was adjusted with a blank solution using the Beckman D.U.-Gilford spectrophotometer. A standard calibration curve was prepared with concentrations of lactic acid ranging from 0 to 9 μg , and its slope⁻¹ ($\mu\text{g}/\text{OD}$) determined. Factors for dilution and slope⁻¹ value were used to calculate the concentration of lactate in mg per 160 ml of total media.

Glucose

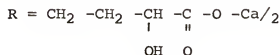
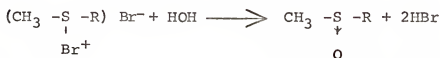
In experiments 1 and 2, glucose was determined by the ferricyanide method of Hoffman (1937). A calibration curve ranging from 4 to 120 mg of glucose was prepared from readings at 420 m μ using the Spectronic-20 photocolormeter.

In the remaining experiments, the Anthrone determination for glucose and/or starch as adapted by Moore et al. (1962) was used. The first steps in the procedure were not followed as insoluble carbohydrates were absent. Reactions were carried out on 1 ml of standard solutions or 1 ml of sample with 2 ml of Anthrone reagent, and the OD read at 625 m μ in the Beckman D.U.-Gilford spectrophotometer. Calculations were based on slope⁻¹ of standard curve and dilution values of glucose were expressed as mg per 160 ml of total media.

Methionine Hydroxy Analog

The method used was based on an adaptation made by the Dupont Company (R. B. Bishop, Personal Communication) of the procedure proposed in "Qualitative Organic Analysis via Functional Groups" (Siggia, 1963) for the assay of sulfide groups in organic compounds. The principle is based on the oxidation of the sulfide to sulfoxide with bromine. Some details will be given here as no fully published account of the method is available. A weighed amount of dry MHA (to

determine degree of purity) or 2 ml of supernatant (to determine the concentration of MHA in the fermentation media) were dissolved in a mixture of acetic acid, water, and hydrochloric acid, 37:10:3 by volume and titrated with a 0.1 N bromide-bromate solution. The end point is a "dead stop" type in which the electrodes are depolarized at the end point by excess bromine. The equations for the bromide-bromate reagent reaction and for the reaction with MHA are as follows:



The oxidation of sulfide to sulfoxide with bromine is a rapid reaction but if an excess of brominating reagent is present the reaction will go to the sulfone and cause high results. The addition rate for the bromide-bromate reagent

is therefore very important in this procedure. The Beckman H-2 pH meter with glass and platinum electrodes was used with a magnetic stirrer. Vortexing was avoided because exposure of the electrodes to air causes redox changes. Using a microburet, the rate of titration was controlled to about 2.5 ml of bromate solution per minute until about 6 to 7 ml were added. The titration rate was then reduced to no more than one small drop per second until the needle suddenly moved through two or more pH units upon addition of one drop of the reagent.

The bromate-bromide solution was prepared by dissolving 283 g of K-bromate and 12 g of K-bromide in water diluting to 1000 ml and storing in a dark bottle. This reagent was checked for normality by titration against 0.1 N sodium thiosulfate using a starch solution as the end point indicator in the presence of 10 per cent KI. To make this titration, about 40 ml of the Br^- solution was pipetted into an iodine flask, 10 ml of 10 per cent HCL added and cooled to 0 - 5 C after which 10 ml of fresh KI solution was added. The flask was then liquid sealed until the solution came to room temperature.

The titration with thiosulfate was carried out to a pale yellow color and then 1 ml of starch indicator was added and titrated until the color disappeared.

Volatile Fatty Acids (VFA)

The supernatant was analyzed for VFA by gas-liquid chromatography. An F and M flame ionization gas chromatograph was used. The column in this instrument contained a stationary phase of Wilken's "free fatty acid polymer" imbedded in an inert support of acid-washed chromosorb W and dimethyldichlorosilane. Nitrogen was the carrier gas, and air and H_2 were the combustion gases for the flame ionizer burner.

A standard mixture of five VFA's was used. The concentrations of the individual acids in the standard, in mM per liter were: acetic 70, propionic 20, butyric 15, isovaleric 3, and valeric 3. This standard mixture was injected after each series of 5 to 10 runs of unknowns and all peak values for each acid were averaged for use in the calculations.

Usually 10 μ l of unknowns as well as standard were injected for each analysis. Ortho phosphoric acid was the acidifying agent in the standard solutions; it was not necessary to add more acid to the unknowns.

The area under each peak was integrated by triangulation, multiplying the height of the peak by the width at the middle distance between the vertex and the base. This was necessary because in the presence of glucose two extra peaks were formed which partially contaminated the acetic and

propionic acid peaks. The areas obtained for the standards were multiplied by the attenuation value divided by 8 ("attenuation" expressed as multiples of 8). This product was then divided by the number of $\text{mM} \times 10^{-6}$ present in 10 μl of standard injected. These ratios were called F values. All F values obtained for each acid during one day were averaged to arrive at an $\bar{F}$ value.

The "area $\times$ attenuation/8" corresponding to each acid of the unknown was multiplied by the corresponding $\bar{F}$ to obtain the number of $\text{mM} \times 10^{-6}$ of each particular acid present in 10 μl of sample injected.

Raw Cellulose

Throughout this work Solka Floc was used as purified cellulose. The material was homogenized in basal medium with a Waring blender to assure uniformity of aliquots for use as initial substrate and for later analysis of residual cellulose.

The method utilized was based on the Crampton and Maynard (1938) procedure with some modifications.

The samples to be analyzed were in the same 50 ml thick-wall centrifuged tubes used to determine DM. Fifteen ml of acetic-nitric acid (5:1) mixture was added to each sample and placed in boiling water.

The subsequent washings with hot water, ethanol, benzene, and ether were done adding the solvents to the tubes, the cellulose dispersed and centrifuged, and the solvents withdrawn by gentle suction. The residual matter was dried at 110 C overnight, cooled and then weighed. All steps were carried out in the same tube.

Ashing and subtraction of the ash weight was omitted because cellulose and bacteria were sedimented together, and N in this dry material was determined. Since low-ash purified cellulose was used in all cases, the error made by omitting ashing is of no serious consequence to the results.

It was decided to call the material analyzed for in this fashion "raw cellulose." Dry matter in the cellulose experiments was composed of raw cellulose and bacterial cells. Another method to determine raw cellulose was to calculate the bacteria weight and subtract it from DM. An estimate of the bacteria weight was calculated by multiplying the N content of the DM by 10, since 10 per cent is the average N content of mixed rumen bacteria cultured in vitro according to Block et al. (1951), Smith and Baker (1944), and from this study.

Results obtained using the two procedures were not significantly different ($P < .1$) by regression analysis. Data

reported on cellulose digestion in this work was calculated using the latter procedure. Cellulose values for time zero were the average of determinations made immediately after inoculation of the media.

Raw Starch

Per cent starch digestion was calculated in the same manner as cellulose digestion. Purified starch devoid of N was used.

The DM in the starch experiments was composed of starch and bacterial cells; therefore, N was determined in the DM, multiplied by 10, and this value subtracted from DM to obtain an estimate of raw starch. No correction for ash was made.

Determinations were carried out immediately after incubation and the average used as initial raw starch in order to calculate per cent starch digestion.

Some Methods Evaluated in a Preliminary Manner

The in vitro methods of this dissertation were adopted after a number of preliminary efforts were made with other procedures. While these preliminary efforts were in some cases not pursued very extensively, it was believed that

sufficient observations were made to describe them.

Among techniques that were considered and failed to be very adaptable were: (1) direct microscopic bacterial counts, (2) colony counts, (3) progressive labeling of the protoplasmic mass with ^{32}P , (4) gas production by bacterial activity, (5) changes in pH, and (6) protein synthesis as measured by the Lowry method. Since no data on these preliminary parts will be presented, only a comment will be made as to the convenience of the techniques used in the present work when compared to those listed above. Such techniques were considered not adaptable to follow in vitro rumen bacterial growth due to factors such as: (a) too much time required as in the case with bacteria or colony counting, (b) excessive handling of the inoculum which may cause population selection as would be the case when depleting the medium of P to raise the specific activity of ^{32}P , (c) inaccuracy of the procedure as in the case of gas or pH measurements, and (d) unsolved technical problems as in the case of extracting and solubilizing all of the bacterial protein for the Lowry colorimetric determination.

In Vivo Procedures

Animals and Experimental Plan

Six adult Florida native sheep with ruminal fistulae were randomly assigned to two groups of three animals each and housed in individual pens. All animals were fed the same ration during three weeks of adaptation at the end of which 2 g of MHA per day were mixed in the daily ration (calculated from the adaptation period) of three of the sheep. The other three animals continued to receive unsupplemented ration. Rumen fluid (RF) and blood samples were taken from the six animals on days 3, 12, and 18 after commencing MHA administration.

All animals were fed at a digestible energy intake equivalent to their maintenance requirement (DE_m), calculated from their body weight (W_{kg}) as: $DE_m = 138 \times W_{kg}^{3/4}$. One kg of ration, made up of ingredients listed below, provided the protein, digestible energy (DE), and mineral elements necessary to meet the animal's requirement (Crampton and Harris, 1969). Water was offered ad libitum.

<u>Ingredients</u>	<u>g</u>	<u>Daily Ration Composition</u>	
		<u>Digestible</u> <u>protein g</u>	<u>D E Kcal</u>
Bermuda grass hay	180.5	6.7	336
ground corn ears	526	30.5	1573
solv. extr. cotton			
seed meal 41%	233	77.3	636
sugar cane molasses	50		119
trace mineral salt	.5		
defluorinated rock			
phosphate	10		
Total	1000.0	114.5	2264

Protozoa Counts

Samples of rumen contents were taken with a small tube-plug device, as described above, just prior to feeding and 4 hours after feeding. Several samplings were taken at different depths and locations within the rumen and combined to make up the rumen fluid sample for each animal. The fluid was immediately strained through four layers of cheesecloth. Protozoa counts were done similar to the method of Warner (1962). One ml was taken under active manual stirring, transferred to a 25 ml volumetric flask, 1 ml of Lugol's solution was added and made up to volume with artificial McDougall's saliva. One drop of this dilution obtained under CO₂ bubbling to insure active stirring was placed on each of the compartments of a hemocytometer and a cover glass placed on top. In this way, the largest protozoa were found lying flat. Each of the nine 1 mm x 1 mm x 0.1 mm volumes

of each compartment was counted and the number of protozoa in the 0.9 mm^3 calculated by averaging the two compartments. These figures were corrected for dilution and finally expressed as number of protozoa per ml of strained rumen fluid.

Bacterial Nitrogen

It was found that when strained rumen fluid was used to determine bacterial N by adding tungstic acid (TA) and centrifugation at 2000 RCF, the supernatant layer remained turbid due to incomplete precipitation of nitrogenous matter. Dilution of 10 ml of strained rumen fluid with 20 ml of water and then addition of TA and centrifugation brought about total precipitation of nitrogenous matter, but the amount of dry matter precipitated was increased about 10 fold over the expected values. This increase in DM was due to a white sediment that also formed from letting a mixture of 20 ml of water and 10 ml of TA stand overnight. It was found that 20 ml of artificial saliva in the presence of 10 ml of TA did not form this sediment when allowed to stand; consequently, the following procedure was adopted.

Fresh strained rumen fluid was centrifuged at 250 RCF for 10 minutes to sediment protozoa and feed particles. Ten ml of the supernatant (SRF_{250}) was transferred to a weighed centrifuge tube, diluted with 20 ml of artificial

McDougall's saliva, 5 ml of sodium tungstate, and 5 ml of 1.07 N H_2SO_4 (TA) added. After standing for at least 4 hours, the total proteins were sedimented and washed two times by resuspending in water and centrifugation at 2000 RCF for 20 minutes. The supernatant and washings were discarded and the residue dried at 60 C in a vacuum oven. Dry matter (DM) was calculated by weighing each tube and its content and subtracting the empty tube weight. Total N determinations were done on the dry residue by the micro-Kjeldahl method digesting directly into the centrifuge tubes. N found in the SRF_{250} sediment was interpreted to be proportional to the bacterial protein concentration. This assumes that before feeding no feed protein was present and that 4 hours after feeding the feed protein would have been rendered soluble in tungstic acid (Borchers, 1965).

Lipoproteins

About 10 ml of blood was taken by jugular puncture from each sheep. Approximately 5 ml of serum was recovered by centrifugation of the blood, diluted with KBr solution to a density of 1.0263, and centrifuged at 50,000 rpm in the Spinco model L preparative-ultracentrifuge for 24 hours. The very low density lipoproteins (VLDL) fraction that separates at the top of the tube was isolated with a needle

and syringe. The density of the remaining serum protein solution was adjusted to 1.070 by adding KBr. The protein fraction that floated after centrifugation of this solution at 50,000 rpm for 24 hours was collected in the same manner. It was designated the low density lipoproteins (LDL).

The density of the remaining protein solution was then increased to 1.208, centrifuged at 50,000 rpm for 24 hours and the high density lipoproteins (HDL) which now floated were collected in the above manner. All fractions were washed once by refloatation in the corresponding density solutions of KBr and transferred to dialyzing bags. Dialysis was done against a phosphate buffer, pH = 7 at 4 C for 48 hours. The individual fractions were lyophilized in the Virtis Lyophilizer.

Total amounts of each individual fraction collected were calculated on duplicate samples by the Lowry method. These values may reflect the relative proportions of lipoproteins but cannot be taken as absolute measurements since recovery may not be complete. Each protein fraction was hydrolyzed in a nitrogen atmosphere at 100 C in 6 N HCl for 24 hours. Amino acid analysis was performed on the hydrolyzates with the use of the Beckman automatic amino acid analyzer.

CHAPTER IV

IN VITRO FERMENTATIONS WITH GLUCOSE AS SUBSTRATE-- EXPERIMENTAL PLAN, RESULTS, AND DISCUSSION

Effect of MHA on the Growth of Rumen Bacteria Cultured with Glucose and Urea

Eight incubation bottles were equally divided into MHA-supplemented and control treatment groups. MHA was added to saturate the basal medium. The concentration obtained was 7.78 mg MHA per ml of total medium. To each fermentation bottle, approximately 1 g of glucose was added. The inoculum may have contained, in addition to bacteria, a few small protozoa, some ammonia, and organic as well as inorganic sulfur (in concentrations not possible to control under our experimental conditions). In addition to the sulfur from the inoculum, MgSO_4 was present in the artificial saliva as part of the basal medium. Consequently, this study measured the effect of adding MHA to a medium generally considered to be adequate for in vitro rumen bacteria fermentations.

One 20 ml aliquot was withdrawn from each bottle at 0, 3, 6 and 13.5 hours of incubation. Results presented in

Table 2 are averages of four determinations at each incubation time, and correspond to bacterial DM, N, and per cent glucose digested. Figure 2 and Figure 3 depict the changes in N and glucose respectively.

No significant differences occurred during the first 3 hours of incubation. At the end of 6 hours the MHA-containing medium had more ($P < .01$) TA insoluble DM and bacterial N. More glucose ($P < .01$) was also digested with MHA. At 13.5 hours of incubation a decrease of DM and protein N was observed in tubes that contained MHA while these substances in the control tubes had continued to increase slightly compared to 6 hours values.

Differences due to treatments were not significant at 13.5 hours of incubation. Cessation of growth after 6 hours with MHA was apparently due to exhaustion of glucose; lack of glucose would be expected to result in a loss of bacterial DM and N due to cellular breakdown.

Glucose disappearance was highly correlated with protein synthesis; $r = .93$ for control $r = .96$ for MHA. No reduction in the concentration of MHA was detectable after any of the fermentation periods.

Table 2

Effect of MHA on Glucose Digestion, Bacterial Dry Matter (DM)
and Bacterial Nitrogen (N) Production at
Increasing Incubation Times

Incubation Time Hours	Control ¹	MHA ¹
DM mg per bottle ²	62.4	56.0
0 N mg per bottle ²	2.32	1.92
Glucose % Dig.	0	0
DM	54.4	88.8
3 N	3.44	3.44
Glucose % Dig.	13	11
DM	157.6 ^A	397.6 ^B
6 N	6.08 ^A	14.64 ^B
Glucose % Dig.	55 ^A	100 ^B
DM	272.8	271.2
13.5 N	9.52	8.40
Glucose % Dig.	100	100

^{AB} Same letter or no superscript indicate no significant ($P < .01$) difference within the same incubation time and response.

¹ Averages of four determinations.

² Incubation bottle, 160 ml of medium.

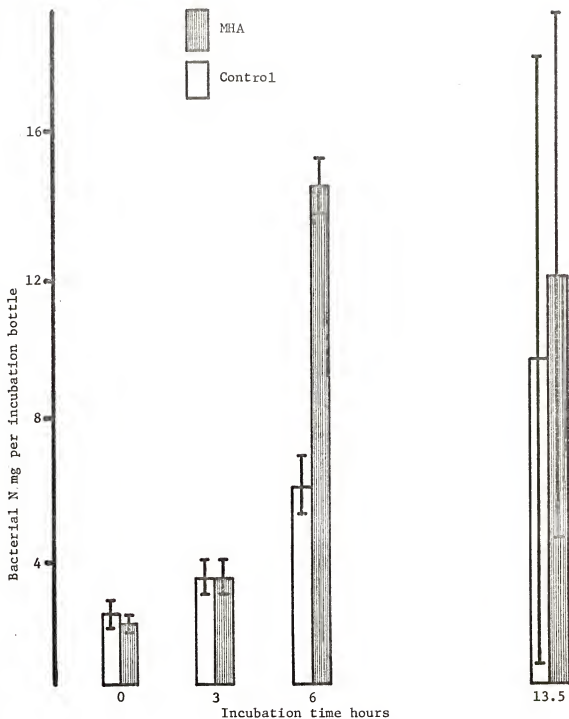


FIGURE 2. EFFECT OF MHA ON IN VITRO RUMEN BACTERIAL PROTEIN N SYNTHESIS AT INCREASING FERMENTATION PERIODS. (Each column is the average of four determinations. Vertical insert lines give standard deviations values.)

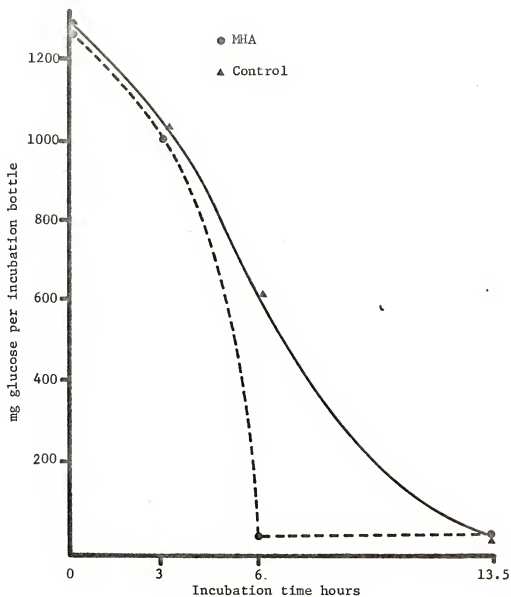


FIGURE 3. EFFECT OF MHA ON IN VITRO RUMEN BACTERIAL UTILIZATION OF GLUCOSE AT INCREASING FERMENTATION PERIODS. (Each plotted point is the average of four determinations)

Effect of Glucose Concentration on Growth
of Rumen Bacteria in the Presence of MHA

Trial 1

The purpose of this experiment was to study the effect of 2 g of glucose in the fermentation medium on the rate of bacterial DM and N synthesis, with and without MHA.

Four fermentation bottles, two with and two without MHA, contained 1 g of glucose. Another four fermentation bottles contained 2 g of glucose, two with and two without MHA. Duplicate aliquots were taken at 0, 4.5, 7, and 10 hours and evaluated for TA insoluble DM, N content of the DM, and glucose present in the media.

Statistical analysis of variations due to treatments was made on the protein N values by the student t test (Dixon and Massey, 1964). The effects of MHA on bacterial N production and glucose utilization with varying times of fermentation are presented in Figures 4 and 5 respectively. There was an increase ($P < .01$) in bacterial N with 2 g glucose after 7 hours of incubation with MHA. By 10 hours of fermentation, greater ($P < .01$) protein N synthesis had occurred with 2 g of glucose with both the MHA and control media. Ten hours were approximately the end of the logarithmic growth phase for the MHA supplemented microbes even though that of control ones was still in progress.

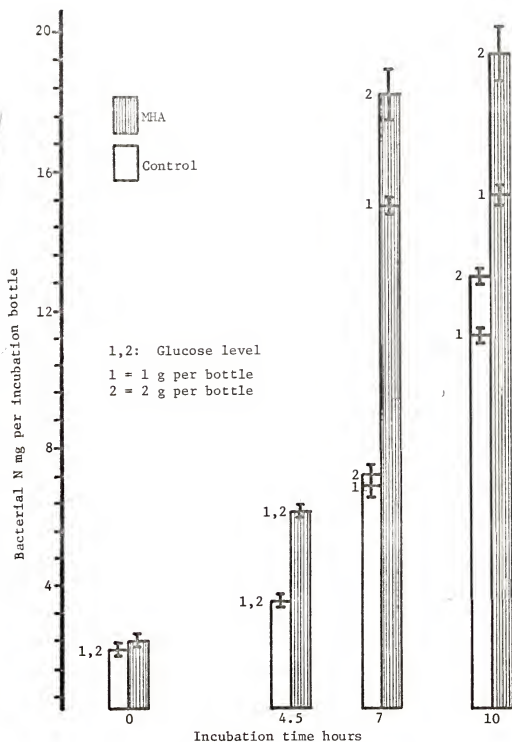


FIGURE 4. EFFECT OF 1 OR 2 g OF GLUCOSE PER INCUBATION BOTTLE, EACH LEVEL WITH AND WITHOUT MHA, ON IN VITRO BACTERIAL N PRODUCTION AS FUNCTION OF TIME

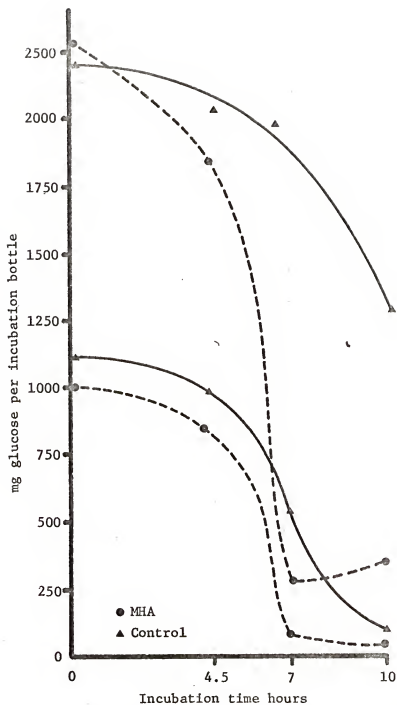


FIGURE 5. EFFECT OF MHA ON GLUCOSE UTILIZATION WHEN 1 OR 2 g OF GLUCOSE ARE PRESENT PER BOTTLE, AS FUNCTION OF INCUBATION TIME

The rate of glucose utilization (Figure 5) was markedly increased when MHA was present during 7 hours of fermentation. When 1 g of glucose was added to the medium essentially all of it was digested in 7 hours of incubation in the presence of MHA compared to 42 per cent digested by controls. When 2 g of glucose was present, the controls utilized 429 mg at 7 hours, while with MHA 2022 mg or 4.7 times as much glucose had been utilized. It is apparent that cessation in bacterial growth in the 1 g glucose medium with MHA at 7 hours was due to depletion of the glucose. Cessation of growth in the 2 g glucose medium with MHA was due to other factors as about 250 mg of glucose remained after no further protein synthesis occurred. Analysis of the supernatant after precipitation of bacteria demonstrated no significant decrease in MHA at the various fermentation periods. This indicates that MHA was not metabolized as a source of energy and that the 0.8 per cent concentration used in this experiment is in excess of the bacterial requirement for the observed stimulation in protein synthesis.

Trial 2

In addition to having two levels of glucose (1 and 2 g) superimposed on the MHA and control treatments, the effect of reconstituting the levels of nutrients after 7 hours of

fermentation was studied. Half of the 160 ml of medium initially present remained in the fermentation bottles immediately after the 7-hour aliquot was taken; 0.5 or 1 g of glucose and 0.5 ml of urea solution were added to each bottle.

Micro-Kjeldahl N was determined on the TA precipitated dry matter. The data obtained are presented in Table 3.

MHA in these trials was stimulatory for protein synthesis. In the absence of MHA, little growth took place for the first 10 hours with either 1 g or 2 g of glucose in the medium. Growth occurred only after 14 and 24 hours of fermentation with 1 g glucose and no MHA. At 7 hours of fermentation a slight decrease in N synthesis indicated cessation of growth in the tubes containing 1 g of glucose plus MHA. This was apparently due to depletion of glucose as growth was reestablished upon addition of more glucose after 7 hours. This experiment demonstrated that at approximately 10 hours the end of the lag-growth phase occurred regardless of substrate availability, in this in vitro ecosystem. Two g of glucose appeared to inhibit growth in the control. With MHA this inhibitory action could be overcome. A replicate trial, however, was not done.

Table 3

In Vitro Rumen Bacterial Protein N at Increasing Incubation Times With 1 or 2 g of Glucose, With or Without MHA

Incubation/ Hours	Control		MHA	
	Glucose		Glucose	
	<u>1g</u>	<u>2g</u>	<u>1g</u>	<u>2g</u>
	Bacterial N mg per incubation bottle			
0	1.68	1.52	1.84	1.60
3	1.52	1.28	7.04	5.60
4.5	1.20	1.36	9.60	11.20
	Reconstituted Media ¹			
7	2.00	2.08	8.40	13.36
10	2.56	1.92	15.20	13.92
14	7.04	2.00	14.64	17.92
24	13.52	3.84	14.56	18.32

Note: Each figure is the average of two determinations.

¹Initial glucose concentrations of 1 or 2 g per 160 ml, plus urea, were reestablished after the 7 hr aliquot taken.

Effect of Different Concentrations of MHA on Growth
and Amino Acid Composition of Rumen Bacteria Cultured
on Glucose

It was believed that the 0.8 per cent MHA concentration in the total medium in previous experiments was in excess of the bacterial requirements.

To define MHA requirements, a portion of the basal medium was saturated with MHA and 0, 2, 5, 10, 30, 70 ml quantities of this saturated solution placed in incubation bottles. Each bottle was then made up to 70 ml with basal medium, and inoculated with 10 ml of 250 RCF supernatant. In this way the following concentrations of MHA were obtained: 0, 0.2, 0.5, 1, 3, and 8 mg/ml of basal medium. Two aliquots were taken from each bottle after 3 and 7 hours of incubation and DM and N in the DM determined. Data obtained are presented in Table 4.

At 3 hours, growth stimulation approached a maximum level with 1 mg of MHA per ml of basal medium. At 7 hours of fermentation 0.2 and 0.5 mg per ml of medium were the best concentrations. These data indicate that concentrations much in excess of the bacterial requirement are inhibitory of the growth response observed, but do not establish at what level of MHA the requirement may be found.

Known amounts of rumen bacteria obtained after 7 hours incubation with glucose and 1 or 8 mg of MHA per ml of basal

Table 4
Effect of Concentrations of MHA on Bacterial Growth

MHA mg/ml	Incubation Time			
	3 hours		7 hours	
	DM*	N [#]	DM*	N [#]
	mg per incubation bottle (160 ml)			
0	25.6	2.88	222.4	14.80
0.2	76.0	5.68	492.8	29.68
0.5	80.8	5.92	468.0	30.16
1	141.6	7.52	432.0	26.64
3	120.8	6.96	410.4	25.28
8	68.8	6.64	380.0	22.00

Note: Each figure is the average of two determinations.

*DM = bacterial DM

#N = bacterial N

medium were treated with 6 N HCl at 100 C for 12 hours in a nitrogen atmosphere for protein hydrolysis. The amino acids (AA) in the hydrolyzate were analyzed with a Beckman automatic analyzer.

The data for AA composition of microbes grown with and without MHA are presented in Table 5. This table also contains for comparative purposes corresponding values reported by Weller (1957) from animals fed three different diets, and values presented by Purser and Buechler (1966).

The AA composition of rumen bacteria in the present study were equivalent when grown at either of the two levels of MHA and for this reason are averaged in Table 5. Some differences occurred in AA composition between values presented by Weller and those of the present study for bacteria grown in glucose medium. Some variations are of about the same magnitude as the differences between the data of Weller and that of Purser and Buechler, but are considerably larger for alanine, arginine, glycine, and tyrosine. These variations are suggestive of differences in the bacterial species that make up the population developed in glucose and that from the rumen.

The data demonstrated that rumen bacteria cultured in the presence of MHA do not contain more methionine.

The possibility of an accumulation of methionine in

Table 5

Per Cent Amino Acid Composition of Rumen Bacteria Grown in Glucose With and Without MHA, and Data from Purser and Buechler (1966) and Weller (1957)

Amino Acid	Control	0.1% and 0.8% MHA	Purser and Buechler	Weller
			b	c
Lysine	6.88	6.92	9.3	8.0
Histidine	.98	1.00	2.3	2.7
Arginine	3.28	3.25	5.4	9.0
Aspartic Acid	10.25	10.47	11.1	6.8
Threonine	6.16	6.14	5.5	3.5
Serine	4.66	4.50	3.8	2.7
Glutamic Acid	11.94	11.97	11.9	6.8
Proline	3.27	3.51	4.1	2.5
Glycine	8.72	9.10	6.1	6.0
Alanine	12.86	12.58	6.5	6.5
Valine	6.30	7.06	6.6	4.4
Methionine	0.48	0.44	2.6	1.5
Isoleucine	6.34	6.35	6.4	3.6
Leucine	8.42	7.53	7.3	4.6
Tyrosine	1.00	1.39	4.2	2.1
Phenylalanine	3.32	3.37	5.1	2.5

a-Averages of the two levels of MHA

b-Averages of 22 strains of bacteria

c-Averages from animals receiving three different diets

the medium due to MHA availability was also investigated. After 7 hours of incubation the bacteria-free supernatant was analyzed for free AA with the Beckman automatic analyzer. No methionine was detected, although traces of lysine, aspartic acid, threonine, serine, glutamic acid, glycine, alanine, and valine were found. The attempt to find methionine was made because some bacterial species have been reported to use N-formyl methionine as initiator for protein synthesis, and subsequently release methionine once the peptide is completed (Watson, 1970). This mechanism has never been shown to be a function in anaerobic rumen bacteria.

Effect of $(\text{MHA})_2\text{-Ca}$, $^1\text{MHA-H}$, ^{1} and DL-Methionine on Growth of Rumen Bacteria and volatile Fatty Acid Synthesis

The Ca salt of MHA binds two moities of B-hydroxy-mercapto-methyl-butyric acid together. Two striking differences exist between methionine (met) and MHA. One is the relative insolubility of MHA, and the other is the lack of an α -amino group in MHA.

The present experiment was designed to test whether

$^1(\text{MHA})_2\text{-Ca}$ and MHA are used here interchangeably; MHA-H refers to compound in the free acid form.

the Ca form and/or the hydroxy group of MHA were essential for the increase in bacterial growth rate and whether there was measurable utilization of the three sources of methyl-sulfide groups and if there was an effect on volatile fatty acid production.

Culturing rumen bacteria in the presence of $(\text{MHA})_2\text{-Ca}$, MHA-H, and DL-met and then comparing the growth responses against an unsupplemented control would provide evidence for chemical groups' functionality as follows: if -OH is essential, $(\text{MHA})_2\text{-Ca}$ and MHA-H, but not DL-met would increase growth rate. If the Ca form is essential, only $(\text{MHA})_2\text{-Ca}$ would enhance growth. If none of the above are essential, all three substances would give the response. Preparation of MHA-H and test of identity of the compound are reported in Chapter III.

Three experimental groups and one control each made up of three fermentation bottles were compared at five incubation periods, i.e., 0, 3, 4.5, 7, and 10 hours. The comparisons were made on DM, protein N, residual glucose, and bromometric values for remaining $\text{CH}_3\text{-S-R}$ (MHA). All experimental conditions remain unchanged except for the introduction of MHA-H and DL-methionine as additional treatment groups.

The initial concentrations of $(\text{MHA})_2\text{-Ca}$, MHA-H , and DL-met were 0.1 per cent of the total media. Results are presented in Table 6. These data indicate that the $-\text{OH}$ group or the Ca in MHA are not essential to increase rumen bacterial growth rate.

Increase in bacterial DM and N are quite linear with time for all four groups. The production of bacterial DM and bacterial N during this linear phase with $\text{Ca}(\text{MHA})_2$, MHA-H , and DL-met supplements in the media was approximately double that of the control ($P < .01$). At 3, 4.5, and 7 hours the three treatment groups were not significantly different from one another. Differences with the control in bacterial DM and N disappeared by 10 hours of fermentation presumably due to exhaustion of glucose.

Bromometric titrations were done to determine the amounts of methyl sulfide groups ($\text{CH}_3\text{-S-R}$) in the $(\text{MHA})_2\text{-Ca}$, MHA-H , and DL-met supplemented media. A 0.01 N bromide-bromate solution was used to insure a high degree of sensitivity. The results obtained are presented in Table 7.

Values corresponding to the three sources of methyl sulfide groups do not differ significantly at any incubation time. Table 7.

Analysis of variance was performed to detect differences between the incubation periods grouping the three

Effect of (MEA)₂-Ca, MEA-H, and DL-Methionine on In Vitro Rumen Bacterial DM and N and Glucose Digestion at Increasing Incubation Times

Incubation Time/Hours	Control		(MHA) 2-Ca		MHA-H		DL-Methionine	
	DM	%Gl ⁺	DM	%Gl	DM	%Gl	DM	%Gl
0	228.0	7.68	225.6	7.60	221.6	7.84	218.4	7.60
		0		0		0		0
3	287.2 ^a	--	502.4 ^b	14.00 ^b	447.2 ^b	14.40 ^b	483.2 ^b	14.48 ^b
		11.09 ^a		23.44 ^b		24.03 ^b		27.67 ^d
4.5	506.4 ^a	13.04 ^a	828.8 ^b	26.24 ^b	900.8 ^b	24.88 ^b	987.2 ^b	29.68 ^b
		18.27 ^a		68.98 ^a		77.49 ^b		85.15 ^b
7	739.2 ^a	20.64 ^a	915.2 ^b	30.40 ^b	1001.6 ^b	32.24 ^b	1001.6 ^b	29.84 ^b
		42.43 ^a		95.63 ^b		97.18 ^b		97.93 ^b
10	1080.0	33.52	888.8	29.68	968.2	27.76	960.0	25.44
		96.20		97.57		97.65		99.10

Note: Each figure is an average of three determinations.

*DM=Bacterial Dry Matter; #N=Bacterial Nitrogen; %G1=Percent Glucose Digestion
 ab. Same letter or no superscript indicates no significant (P<0.01) difference within the
 same incubation time and response.

Table 7

Utilization of $\text{H}_3\text{C-S-R}$ as $(\text{MHA})_2\text{-Ca}$, MHA-H , and
 DL-Methionine by Rumen Bacteria at Increasing
 Incubation Times^{1,2}

<u>Incubation Time/Hours</u>	<u>Control</u>	<u>$(\text{MHA})_2\text{-Ca}$</u>	<u>MHA-H</u>	<u>DL-Methionine</u>
Expressed as mg of MHA per 160 ml of total media				
0	--	106	102	118
3	12	114	108	110
4.5	10	112	101	110
7	10	107	83	86
10	8	89	80	84

¹The methyl sulfide was titrated with Br-BrO_3 reagent and the values calculated were expressed as mg of MHA per 160 ml of media in case of all four treatments.

²Each figure is the average of three determinations.

treatment values together. Results indicated that there was a highly significant ($P < .01$) decrease in titratable methyl sulfide concentration between 4.5 and 7 hours. No other differences between two consecutive periods of incubation were significant although a continuous decrease took place from 3 hours until the end of the incubation time.

Results obtained for VFA's expressed in mM per liter and in per cent molar composition of the acetic, propionic and butyric acids mix at increasing incubation times is presented in Table 8. Comments on these data will be found together with comparable data under the section entitled "Effect of MHA on the Lag Phase and Growth Rate of Rumen Bacteria and Fermentation Products."

The possibility that MHA would increase the growth rate of rumen bacteria by acting as a reducing substance making the medium more anaerobic was tested in this experiment. No precise measurements of redox potentials were attempted due to the inadequacy of the available electrodes. Instead, comparisons of redox power were made using cysteine-cystine, $E^{O'} = 0.340$ volts at pH = 7.0 and 30 C as reference system; and methylene blue, $E^{O'} = -0.011$ volts at pH = 7 and at 30 C as redox indicator. The dye is colored at redox potentials above its $E^{O'}$ and colorless when at lower (more negative) potentials.

Table 8

VFA'S Produced by Rumen Bacteria in Glucose
Medium With and Without MHA₂-Ca, MHA-H, and DL-Methionine

Incubation Time/Hours	VFA	Control		(MHA) ₂ -Ca		MHA-H		DL-Met	
		mM/l	%M ¹	mM/l	%M ¹	mM/l	%M ¹	mM/l	%M ¹
0	C ₂	8.59	67.3	8.30	66.0	11.33	73.6	9.32	66.1
	C ₃	2.31	18.1	2.35	18.7	2.50	17.2	2.58	18.3
	C ₄	1.85	14.5	1.91	15.2	1.56	10.1	2.19	15.5
3	C ₂	11.50	65.4	12.19	67.5	12.91	67.5	12.51	67.2
	C ₃	3.70	20.9	3.53	19.5	3.62	12.9	3.84	20.6
	C ₄	2.41	13.6	2.33	12.9	2.57	13.4	2.24	12.0
4.5	C ₂	11.06	63.7	14.30	71.3	16.04	72.3	14.14	71.0
	C ₃	3.85	22.2	4.56	22.7	4.60	20.7	4.56	22.9
	C ₄	2.44	14.0	1.19	5.9	1.53	6.9	1.19	6.0
7	C ₂	15.46	64.5	14.27	66.4	15.41	66.6	13.55	67.6
	C ₃	5.65	23.6	5.82	27.1	6.38	27.4	5.29	26.4
	C ₄	2.82	11.7	1.37	6.4	1.35	5.8	1.19	5.9
10	C ₂	17.12	59.4	16.86	62.4	17.71	59.7	14.59	59.6
	C ₃	8.00	27.7	8.59	31.8	10.36	34.9	8.61	35.2
	C ₄	3.68	12.7	1.55	5.7	1.54	5.2	1.25	5.1

¹Percent molar composition.

When equal concentrations of cysteine, MHA, or methionine in basal medium and basal medium inoculated with rumen bacteria were checked for redox power with a Beckman potentiometer fitted with a pair of platinum-glass electrodes, the medium with bacteria caused the most negative shift of the needle; cysteine resulted in a less negative shift, and MHA and methionine gave an almost imperceptible shift. This is an indication that rumen bacteria generate lower redox potential than cysteine, MHA or methionine.

A second approach consisted in adding enough drops of a 0.4 per cent methylene blue solution to color a CO_2 equilibrated basal medium (no bacteria) solution, with and without MHA, at $\text{pH} = 6.9$, and 39°C . No color change occurred in any of the bottles after $1/2$ hour of incubation. Then, inoculation with 20 ml of supernatant rumen fluid (250 RCF) were added to 140 ml of basal medium in each bottle and incubation continued. The blue color disappeared from both the MHA-supplemented and the unsupplemented total medium in an average time of 4.5 minutes of incubation.

This indicated that the bacterial activity generates a redox potential lower (more negative) than $E^{\circ'} = 0.011$ volts. It may be concluded that MHA cannot exert a reductive action in medium inoculated with rumen bacteria since

the activity of the microbes generate a lower E^0 than the redox potential of MHA.

Effect of MHA, Sulfur-Containing Amino Acids, Sulfate, and Sulfite as Sources of Sulfur on Growth of Rumen Bacteria

A test was made to determine if the increased growth of rumen bacteria utilizing glucose in the presence of MHA was due to the extra S added to the medium as MHA and whether other sources of S could produce the same effect.

MHA, DL-methionine, cysteine HCl, $MgSO_4 \cdot 7H_2O$, Na_2SO_4 , and Na_2SO_3 were added in amounts equivalent to 10 μg of S per incubation bottle. MHA was also added at a relatively high concentration of 0.1 per cent or 30.29 mg of S per incubation bottle.

Twenty ml aliquots were taken at 0 and 4.5 hours of incubation for determinations of bacterial DM and N and utilization of glucose. The data obtained are summarized in Table 9.

The two levels of MHA and the S containing AA, when added to the medium, resulted in bacterial DM and N production two to three times greater than occurred in the bottles that had equal amounts of S as inorganic salts. The three inorganic sources of S did not differ significantly from the control values in any of the responses measured.

Table 9

Effect of Added Organic and Inorganic Sources
of S on Bacterial Dry Matter (DM) and Bacterial N
Production and Glucose Utilization,
at 0 and 4.5 Hours of Incubation

	Incubation Time/Hours					
	0			4.5		
	DM	N	Gluc.	DM	N	Gluc.
mg per incubation bottle (160 ml)						
$\bar{x}_4$ Control				177.6	8.24	1000
S.D.				8.8	.24	112
$\bar{x}_4$ $\text{Na}_2\text{S}_2\text{O}_3$	$\uparrow$	$\uparrow$	$\uparrow$	171.2	8.32	1120
S.D.				16.8	.32	154
$\bar{x}_4$ Na_2SO_4	$\uparrow$	$\uparrow$	$\uparrow$	168.9	7.92	924
S.D.				13.6	.08	165
$\bar{x}_4$ MgSO_4				151.2	8.16	1156
S.D.				20.0	.40	156
$\bar{x}_4$ DL-Met				464.8	21.60	392
S.D.				27.2	1.04	49
$\bar{x}_4$ Cysteine	$\bar{x}_{16} = 84.8 \pm 5.9$ (S.D.)	$\bar{x}_{16} = 4.08 \pm .14$ (S.D.)	$\bar{x}_{16} = 1002 \pm 180$ (S.D.)	569.6	26.32	216
S.D.				23.2	1.28	20
$\bar{x}_4$ MHA	$\downarrow$	$\downarrow$	$\downarrow$	362.4	15.52	528
S.D.				21.6	1.28	119
$\bar{x}_4$ MHA 0.1%	$\downarrow$	$\downarrow$	$\downarrow$	425.6	19.84	428
S.D.				24.0	.72	92

Concentrations of treatment substances are equivalent to 10 μg of S per bottle, except for MHA 0.1%, equivalent to 30 mg of S per bottle.

Glucose was utilized in amounts relative to the DM and N synthesized as shown by data recorded at 4.5 hours of fermentation.

The 0.1 per cent level of MHA, when compared to the lower MHA level, caused a higher ($P < .01$) bacterial DM and N production at 4.5 hours, but only slightly more glucose was utilized.

Effect of Eighteen Amino Acids and MHA on Growth of Rumen Bacteria

Eighteen amino acids and MHA were tested for their effect on bacterial DM and N production as well as glucose consumption during 4.5 hours of incubation. Each AA was tested separately at a concentration of 0.31 m moles per ml of medium. MHA was added at the level of 0.15 m moles of the Ca salt. These concentrations provided approximately 10 μ g of S per ml of medium.

Averages of two determinations for the effect of each amino acid on bacterial DM and protein N and four determinations for the control medium are presented in Table 10. The S-containing amino acids and MHA increased bacterial DM and N to approximately twice that of the control, and stimulated glucose consumption approximately three times more than that in the control group.

Table 10

Effect of Amino Acids and MHA on Rumen Bacterial DM
and Bacterial N Production and Glucose Utilization

	Incubation Time; hours				
	0		4.5		$\bar{x}_{2-4}$
	DM	N Gluc.	DM	N	Gluc.
	mg per incubation bottle (160 ml)				
Control			435.2	18.40	792
Val.			467.2	19.60	499
Phe.			444.8	18.96	738
Ala.			447.2	20.32	577
Ser.			470.4	20.32	558
Glu.			500.0	21.20	574
Pro.	$\bar{x}_{20} = 216.0 \pm 9.0$ (S.D.)	$\bar{x}_{20} = 9.44 \pm .228$ (S.D.)	$\bar{x}_{20} = 1068 \pm 146$ (S.D.)	20.64	617
Leu.				21.60	535
Thr.				20.48	596
Ile.				19.28	802
Arg.				20.80	574
Asn.				20.88	717
Asp.				21.04	689
Lys.				20.48	613
Tyr.			487.2	21.92	638
His.			497.6	21.04	796
Cys.			708.0	28.96	210
Met.			745.6	31.68	66
Cystine			738.4	34.24	120
MHA			724.8	32.96	138

The other amino acids caused a slightly higher production of bacterial DM and N. This slight increase of metabolic activity was also indicated by slightly increased glucose utilization.

Effect of MHA on the Lag Phase and Growth Rate of Rumen Bacteria and Fermentation Products

Each of four incubation bottles contained 0.15 m moles of MHA per 160 ml of medium. Treatment and equal number of control bottles were aliquoted each 0.5 hour intervals from 0 to 8 hours of fermentation.

Bacterial DM and N were determined and the supernatants were analyzed for glucose, ammonia N, volatile fatty acids

Values for bacterial DM (Figure 6), bacterial N calculated by two different methods (Figure 7), and glucose utilization (Figure 8) involved more observation than previous similar trials when fermentation media were sampled at longer intervals. These values and those of previous ones demonstrated the same magnitude of MHA stimulation.

As shown in Figure 9, ammonia N in the medium began to increase linearly immediately upon starting incubation. This indicated that the inoculum bacterial urease is quite effective for urea hydrolysis and that ammonia was not a limiting factor during the lag phase of bacterial growth.

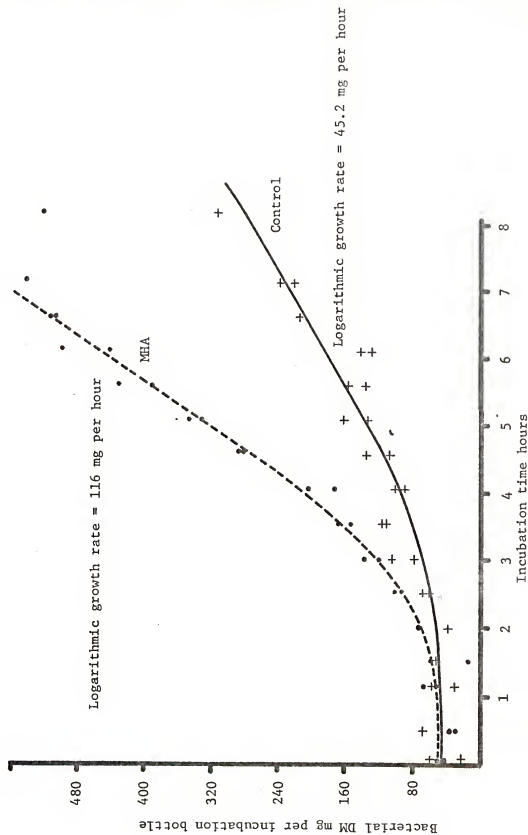


FIGURE 6. RUMEN BACTERIA GROWTH CURVE, EXPRESSED AS BACTERIAL DRY MATTER (DM) PRODUCED AS FUNCTION OF INCUBATION TIME IN A MEDIUM CONTAINING GLUCOSE AND UREA WITH AND WITHOUT MHA

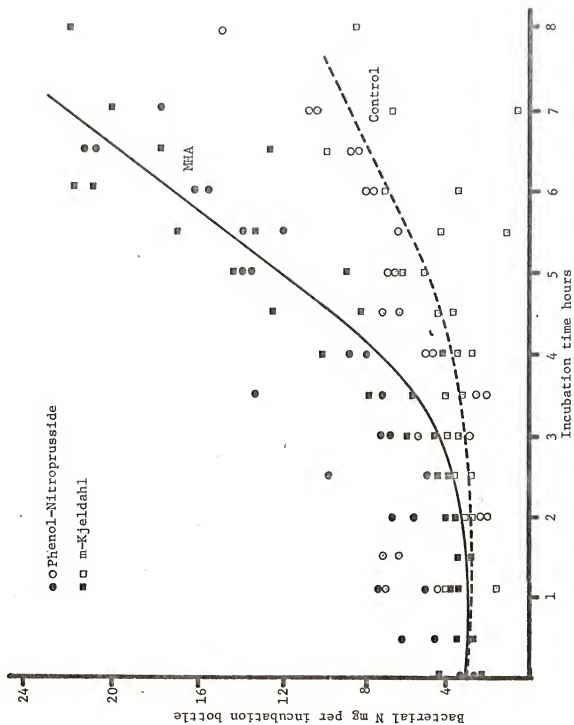


FIGURE 7. RUMEN BACTERIA GROWTH CURVE EXPRESSED AS BACTERIAL N PRODUCED AS FUNCTION OF INCUBATION TIME IN A MEDIA CONTAINING GLUCOSE AND UREA WITH AND WITHOUT MHA. BACTERIAL N ASSAYED BY TWO METHODS

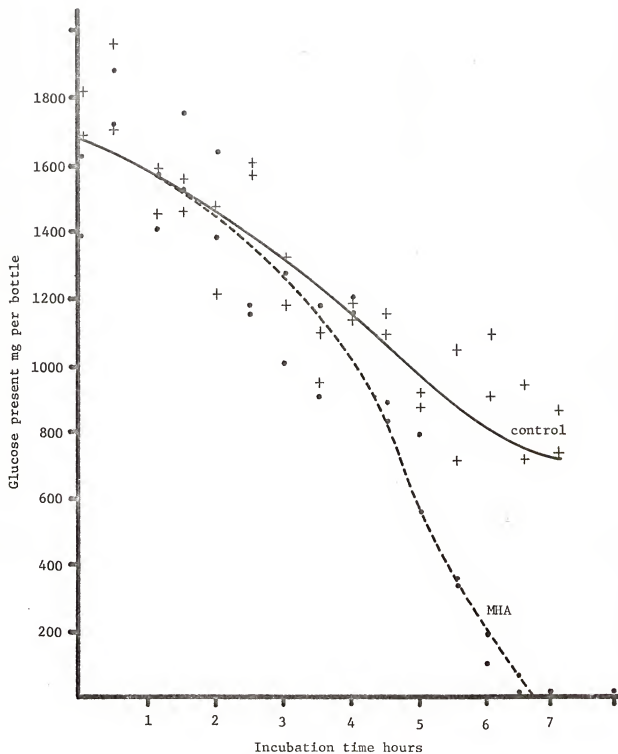


FIGURE 8. GLUCOSE UTILIZATION BY R. BACTERIA AS FUNCTION OF INCUEATION TIME IN MEDIA WITH UREA WITH AND WITHOUT MHA

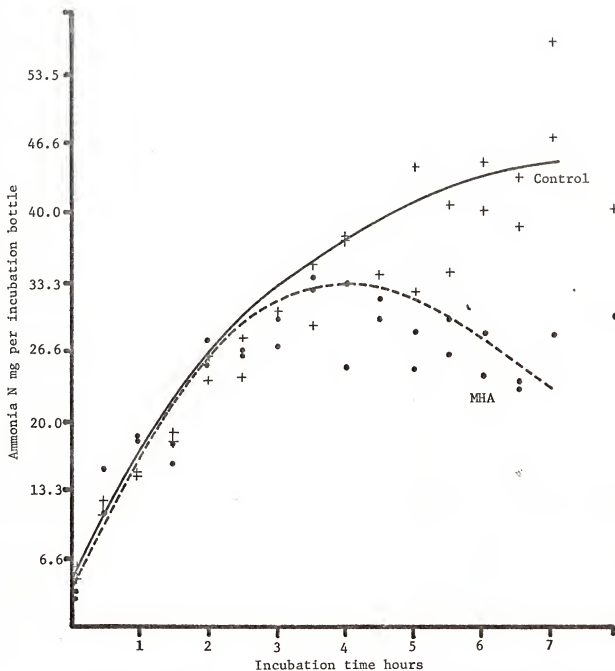


FIGURE 9. AMMONIA FROM UREA AS FUNCTION OF INCUBATION TIME BY RUMEN BACTERIA IN MEDIUM CONTAINING GLUCOSE, WITH AND WITHOUT MHA

Ammonia concentration increased at the same rate for the MHA and control groups during the first 3 hours of incubation and demonstrated a faster rate of urea hydrolysis than ammonia utilization. Urea in the medium of the MHA group appeared to reach complete hydrolysis by 4 hours. After this time, ammonia concentration decreased in the MHA medium which was coincident with the higher growth rate taking place. The ammonia concentration in the control group increased after 4 hours of incubation but at a slower rate.

Concentrations of acetic (C_2), propionic (C_3), and butyric (C_4) acids and molar per cent contributed by each acid to the total mix of VFA's were determined at 0, 3, 4.5, 7, and 10 hours of fermentation.

Values for the present experiment are presented in Table 11. They agreed generally with data of experiment 5 (Table 8). In both experiments, the concentration of each individual acid increased with time of incubation, but C_3 increased at a faster rate so that the molar per cent composition of the total VFA at each progressing sampling time showed a decrease for C_2 and an increase for C_3 , i.e., $C_2/C_3 = 4.6$ at 0 hours and $= 2.3$ at 8 hours (Table 11). Only a slightly greater production of m moles of VFA took place in the MHA medium even though the microbes approximately

Table 11

VFA's Produced by Rumen Bacteria in Glucose Medium
With and Without MHA

Incubation Time/Hours	VFA	Control		MHA	
		mM/l	%M ^a	mM/l	%M ^a
0	C ₂	6.56	71.45	6.56	71.07
	C ₃	1.54	16.77	1.64	17.76
	C ₄	1.08	11.76	1.03	11.15
3	C ₂	8.65	72.02	8.65	70.56
	C ₃	2.37	19.73	2.40	19.78
	C ₄	0.99	8.24	1.08	8.90
5	C ₂	9.41	66.24	11.12	68.43
	C ₃	3.33	23.41	3.69	22.70
	C ₄	1.48	10.40	1.44	8.86
6	C ₂	11.41	68.56	12.24	67.10
	C ₃	3.75	22.53	4.52	24.78
	C ₄	1.48	8.89	1.48	8.11
7	C ₂	12.13	64.01	12.90	64.75
	C ₃	4.58	24.16	5.63	28.26
	C ₄	2.24	11.82	1.39	6.97
8	C ₂	13.12	64.53	13.73	64.60
	C ₃	5.54	27.25	6.34	29.76
	C ₄	1.67	8.21	1.23	5.77

^apercent molar composition

doubled their mass compared to those without MHA. However, lactic acid concentration in the MHA medium was greater as presented in Figure 10. The rate of lactate production was 300 mg per hour in the presence of MHA compared to 65 mg per hour in its absence. This is a 4.6 fold increase in lactic acid production due to MHA in medium where bacterial growth was approximately doubled. The lactate production in the MHA medium ceased after approximately 7 hours of incubation. At this time glucose was depleted.

In order to compare the conversion of Glucose to bacterial cells, lactate and VFA's; bacterial cell weights were calculated as ten times the bacterial N present at 7 hours.

Calculations show that 1650 mg of glucose were converted to 1,080 mg of lactic acid, 220 mg of acetic, propionic and butyric acids and 225 mg of bacterial cells.

Assuming equimolecular conversions, only 125 mg of glucose are not accounted for which could represent volatile products. These figures reveal the prevalence of lactic acid as catabolic product.

The conversion of glucose to bacterial cells was 13.6 per cent which agrees with values cited in the literature ranging from 9.5 to 17.5 per cent (Hungate, 1966, p. 315).

It may be that under conditions of accelerated energy

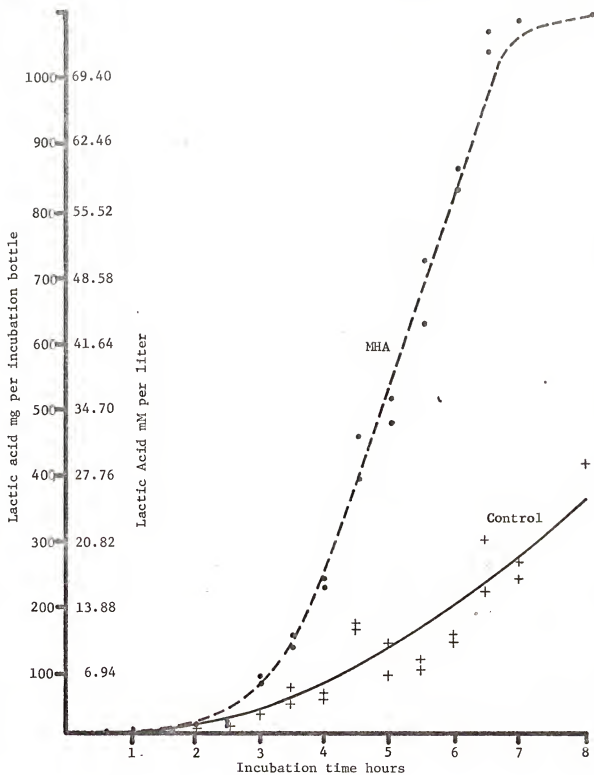


FIGURE 10. LACTIC ACID PRODUCTION BY RUMEN BACTERIA CULTURED WITH GLUCOSE AND UREA, AS FUNCTION OF INCUBATION TIME, WITH AND WITHOUT MHA

utilization by the rumen bacterial cells, the reduction of pyruvate to lactate becomes the main reaction for the oxidation of DPNH necessary for glycolysis. The conversion of pyruvate to C_2 is limited since it requires oxidized DPN^+ , but the formation of C_3 is increased due to the abundance of lactate, some of which is converted to acrylate and finally to C_3 .

Cellulolytic Activity of Rumen Bacteria Grown with Glucose

The majority of rumen bacteria species can utilize glucose (Hungate, 1966b). A possibility may exist that some minor species may utilize only glucose. If such were the case, those species would rapidly populate the glucose medium and a shift of population from the common species of the rumen would result.

Such species may not synthesize methionine fast enough and the observed MHA growth stimulation with glucose as the source of energy could be of little consequence to the rumen bacteria.

An attempt was made to obtain information on this question by preparing gram stains of fresh rumen microbes and corresponding ones after incubation in medium with glucose, with and without MHA. No major differences in morphology or staining of the bacteria could be observed.

Microscopic similarity is not proof of species identity.

A test was made for cellulolytic activity of rumen bacteria grown in glucose medium prior to inoculation on cellulose. Rumen bacteria were cultured in glucose medium for 6 hours with and without MHA.

Twenty ml of such suspensions of microbes were used to inoculate three fermentation bottles containing 2 g of purified cellulose in 140 ml of medium supplemented with MHA at levels of 7, 50, and 100 mg per 160 ml. Twenty ml of bacterial suspension without MHA were used to inoculate a fourth bottle without MHA. Four other bottles were inoculated with 20 ml of fresh supernatant (250 RCF) rumen fluid. These eight bottles were incubated and duplicate 20 ml aliquots taken from each at 24- and 48-hour intervals. Determinations done included total DM, and raw cellulose in the DM. Average values for per cent raw cellulose digested are presented in Table 12.

No significant differences resulted from the three levels of MHA supplementation and for this reason the individual observations were averaged as shown in Table 1. Levels of MHA higher than 7 mg per 160 ml of total medium (0.8 per cent) were not necessary for the increase in growth rate in both glucose and cellulose media.

Differences in cellulose digestion with and without

Table 12

Cellulolytic Activity of Rumen Bacteria Grown in
Glucose, Compared to Fresh Rumen Fluid Activity,
With or Without Three Levels of MHA

Per cent Cellulose Digestion		
<u>MHA mg per 160 ml</u>	Inoculum	
	<u>Bacteria Grown in Glucose¹</u>	<u>Supernatant² Rumen Fluid²</u>
24 hours of incubation		
0	10	40
7, 50, or 100 ³	10	42
48 hours of incubation		
0	51	76 ^A
7, 50, or 100 ³	46	89 ^B

^{AB} Different capital letters indicate a significant ($P < .01$) difference between consecutive figures in a column.

¹ Inoculum was 20 ml of microbes suspension after incubation in a glucose medium for 6 hours.

² Inoculum was 20 ml of supernatant solution from fresh rumen fluid after centrifugation at 250 RCF for 10 min.

³ Results from three levels of MHA were averaged.

MHA at 24 and 48 hours of incubation were analyzed by t test. There was an increase in cellulose digestion ($P < 0.1$) within 24 hours when rumen fluid was used as inoculum. Bacteria grown on glucose did not produce this effect. After 48 hours cellulose fermentation was greater ($P < .01$) with MHA when rumen fluid was the inoculum, but not with bacteria grown on glucose. Mixed bacteria grown on glucose for 6 hours both in the presence and absence of MHA maintained ability to digest cellulose. The extent of cellulose digestion for bacteria that had never been exposed to high concentrations of glucose was almost 100 per cent by 48 hours compared to about 50 per cent for those exposed to glucose. This difference seems due to a longer lag phase for glucose-exposed bacteria. The per cent of cellulose digested by 24 hours was 10 per cent for glucose-exposed bacteria compared to 40 per cent by bacteria procured directly from the rumen.

CHAPTER V

IN VITRO FERMENTATIONS WITH CELLULOSE AS SUBSTRATE-- EXPERIMENTAL PLAN, RESULTS, AND DISCUSSION

Five trials were made to determine if cellulolytic rumen bacteria benefit from supplementation with MHA, methionine, or inorganic sulfate. The number of treatments and responses measured in each trial varied. In trial 1 the stimulatory action of MHA on cellulose digestion was measured. The concentration of MHA was 0.5 g per 100 ml of basal medium. Two g of purified cellulose were added to each fermentation bottle. As shown in Table 13 average cellulose digestion percentages of 26.3 and 20.5 per cent ($P < .01$) were obtained during 24 hours of fermentation for the MHA and control flasks, respectively. Corresponding cellulose digestion values of 93.8 and 73.3 were obtained at 48 hours, respectively ($P < .01$).

Digestibility of cellulose measured after 12 hours of fermentation was about 3 per cent and not influenced by the MHA.

In trial 2 (Table 13), measurements of DM in the media at 24 and 48 hours of incubation were obtained following

addition of tungstic acid. Two g of purified cellulose were initially added to each bottle. Seven and 100 mg levels of MHA in the medium were tested. Bacterial N was determined in the precipitated DM and ammonia; VFA's and lactate were determined in the supernatant solutions.

The values obtained for the MHA and control media at 24 hours of fermentation were 28.7 and 24.4 per cent for cellulose ($P < .05$), and 24.0 and 18.7 mg per 160 ml medium for bacterial N ($P < .05$), respectively. Corresponding values at 48 hours of fermentation for the MHA and control media were 91.1 and 80.9 per cent ($P < .05$) for cellulose, and 19.3 and 13.9 mg per 160 ml for bacterial N ($P < .05$), respectively. The increase in cellulose digestion due to MHA was directly proportional to the increase in bacterial N at 24 and 48 hours.

Ammonia concentration at 24 hours of fermentation was essentially the same in the control and MHA media, but it was lower ($P < .05$) for MHA at 48 hours. The three responses measured indicated a higher metabolic activity for microbes in the MHA treatment.

A third trial with cellulose as the substrate was carried out to help clarify the following aspects of MHA as a stimulatory agent for cellulolytic microbes: (1) the

comparative effect of MHA and DL-methionine on cellulose digestion, bacterial N synthesis, and ammonia availability from urea, and (2) the effect of MHA and/or methionine in the presence of higher amounts of cellulose substrate in the medium. Purified Solka Floc cellulose was introduced into the fermentation bottles as a 50 ml suspension in artificial saliva as it was mixed in a Waring blender at moderate speed. It was determined that the average amount of cellulose initially present was 3.28 g per bottle.

Volatile FA and lactate were also determined. Averages for results other than VFA and lactate are presented in Table 13.

Cellulose digestion was significantly higher ($P < .01$) in the presence of either MHA or DL-met than in control medium at 24 and 48 hours of fermentation as shown in Table 13. DL-methionine and MHA were not significantly different from each other.

The 40 to 55 per cent cellulose digestion was better in the fermentation trial with 3.28 g cellulose than the 20 to 28 per cent digestion obtained with 2 g of cellulose in experiments 1 and 2, with or without MHA. The difference in cellulose digestion between MHA and control treatments was 15 per cent greater with 3.28 g of cellulose compared to a difference of 5 per cent with 2 g.

At 48 hours the extent of cellulose digestion was greater ($P < .01$) for MHA (98.3 per cent) or DL-methionine (97.2 per cent) than control medium (86.9 per cent).

The effect of MHA or methionine was enhanced for bacterial N production when cellulose was available at the 3.28 g level. Apparently, it was less limiting than at the 2 g concentration per bottle and faster bacterial growth occurred as indicated by the greater amounts of bacterial N observed for trial 3 (Table 13). Values for bacterial N averaged 68.7, 74.4, and 32.6 mg per 160 ml medium at 24 hours of fermentation for the MHA, DL-met, and control bottles, respectively ($P < 0.1$). MHA and DL-met did not differ significantly in their effect.

Corresponding bacterial N values for the three treatments at 48 hours of digestion were 52.0, 65.8, and 46.2 mg per 160 ml, respectively. DL-met values were higher ($P < .01$) than those of either MHA or the control medium. The greater bacterial protein synthesis at 48 hours of incubation with DL-met may have been due to the additional N of methionine. The N supplied by the urea in the medium may have been limiting at 48 hours of incubation as only 5.1 mg per 160 ml medium remained.

In trials 4 and 5 fermentations were carried out to determine whether an inorganic source of sulfur would have

the same effect as MHA and methionine on cellulolytic bacteria.

Each of the three sulfur sources provided 10 μg of S per ml of total medium. The initial cellulose content of each incubation bottle was approximately 3 g.

In trial 4, four incubation bottles were used for control and sulfate treatment groups and two each for MHA and DL-met treatments. Thus, there were eight and four samples respectively at each collection period. In trial 5, three bottles were used for each treatment giving six samples per treatment at each collection time.

Averages for these two trials are presented in Table 13. Least-squares analysis of variance and Duncan's test were performed for trials 4 and 5 together, blocking for trials. Least-squares means for both trials are presented in Table 14. These data indicate that after 24 hours of incubation the per cent of cellulose digested by the MHA and DL-met supplemented bacteria was higher ($P < .01$) than the sulfate and control groups.

MHA stimulated more ($P < .01$) bacterial N production than occurred with the other three treatments. The three supplemented groups had lower ammonia concentration ($P < .01$) after 24 hours of fermentation.

Table 13

Effect of MHA, DL-Methionine, and Sodium Sulfate on
Cellulose Digestion, Bacterial Nitrogen, and
Ammonia Utilization by Rumen Bacteria In Vitro

Trial No.	Incubation Time/Hours	Observa- tions per treat- ment *	Cellulose Digestion			
			Control	MHA	% DL-Met#	Na ₂ SO ₄
1	24	4	20.5 ^A	26.3 ^B		
	48	4	73.3 ^A	93.8 ^B		
2	24	8	24.4 ^a	28.7 ^b		
	48	8	80.9 ^a	91.1 ^b		
3	24	8	39.9 ^A	55.2 ^B	52.2 ^B	
	48	8	86.9 ^A	98.3 ^B	97.2 ^B	
4	24	8-4-4-8	32.5 ^A	41.5 ^B	-34.6 ^A	31.9 ^A
	48	8-4-4-8	63.8 ^A	83.8 ^B	-79.3 ^B	76.1 ^B
5	24	6	20.1 ^A	26.3 ^{Ba}	30.3 ^{Bb}	22.7 ^A
	48	6	54.2 ^A	65.9 ^{Bb}	71.3 ^{Ba}	67.3 ^{Bb}

Table 13 (extended)

Bacterial N mg/bottle**				Ammonia N mg/bottle**			
Control	MHA	DL-Met#	Na ₂ SO ₄	Control	MHA	DL-Met#	Na ₂ SO ₄
18.72 ^a	24.00 ^b			44.5	44.0		
13.92 ^a	19.36 ^b			36.3 ^a	31.4 ^b		
32.64 ^A	68.72 ^B	74.48 ^B		17.7	19.1	19.4	
46.24 ^A	52.08 ^B	65.84 ^C		13.7 ^A	5.2 ^B	5.1 ^B	
26.48	27.84	28.80	24.96	31.2 ^A	21.6	27.6 ^B	29.6
21.84 ^A	25.60 ^B	25.60 ^B	28.56 ^C	24.1 ^A	11.0	11.7 ^B	6.1 ^C
23.60 ^A	29.92 ^B	22.40 ^{Aa}	18.24 ^{Cb}	20.1 ^{Aa}	15.2 ^B	14.9 ^B	17.4 ^A
26.00 ^A	34.72 ^{Ba}	36.72 ^B	37.84 ^{Bb}	16.8 ^A	13.5 ^{Ba}	14.2 ^C	11.5 ^D

ABC Same letter or no superscript indicates no significant (P<.01) difference within the same incubation time and response

ab Idem (P<.05)

*Each is the average of 4, 8 or 6 determinations.

**160 ml of total medium

#DL-Methionine

Table 14

Effect of MHA, DL-Methionine, and Na_2SO_4 on Cellulose Digestion, Bacterial Nitrogen (N) and Utilization of Ammonia N, Measured at 24 and 48 Hours of In Vitro Fermentation by Rumen Bacteria

<u>Incubation Time/Hours</u>	<u>Control</u>	<u>MHA</u>	<u>DL-met</u>	<u>Na_2SO_4</u>
Cellulose digestion %				
24	26.3 ^A	33.9 ^B	32.4 ^B	27.3 ^A
48	59.0 ^A	74.8 ^{Ba}	75.3 ^{Ba}	71.7 ^{Bb}
Bacterial N mg/bottle ¹				
24	25.04 ^A	28.72 ^B	25.60 ^A	21.60 ^A
48	23.99 ^A	30.16 ^{Bb}	31.12 ^{Ba}	33.20 ^{Bb}
Ammonia N ₁ mg/bottle ¹				
24	25.6 ^A	18.4 ^B	21.2 ^B	23.5 ^B
48	20.4 ^A	12.2 ^B	12.9 ^{Bc}	8.8 ^{Bd}

Note: Each figure is the average of 14 determinations obtained from trials 4 and 5.

^{AB} Same letter or no superscript indicate no significant ($P < .01$) difference within the same incubation time and response.

^{ab} Idem ($P < .05$).

¹ 160 ml of total medium.

At 48 hours, the MHA, DL-met, and sulfate supplements caused greater ($P < .01$) digestion of cellulose than the control. The MHA and DL-met were superior ($P < .05$) to sulfate.

Approximately the same treatment effects and levels of significance were obtained for bacterial N as for cellulose digestion at 48 hours, except that sulfate was better ($P < .05$) than MHA and DL-met.

This larger concentration of bacteria in the presence of sulfate at 48 hours was due to decrease in the amount of precipitable cellular N in the MHA and DL-met groups in trials 2, 3, and 4 as cellulose was depleted earlier in these treatments. This was also indicated by the greater cellulose digestion and ammonia concentration associated with MHA and DL-met treatments in experiments 4 and 5 at 48 hours. The DL-met supplemented group had the highest ammonia concentration at 48 hours.

Results from gas chromatography of VFA found in the medium at 24 and 48 hours of digestion of cellulose by rumen bacteria with and without MHA or DL-methionine are presented in Table 15 (trial 2) and in Table 16 (trial 3).

In both trials there was an increase ($P < .01$) of total VFA as well as of each individual acid with increasing time

Table 15

Volatile Fatty Acids mM Per Liter and Per Cent Molar (%M)
 Contribution of Each Acid at Increasing Times of In Vitro
 Cellulose Fermentation by Rumen Bacteria, With and Without
 MHA--Trial #2

Fermt. Time	Acid	Control		MHA	
		mM/l	%M	mM/l	%M
24 hrs. $\bar{x}_4$	C ₂	12.16	63	13.59	61
	C ₃	6.13	33	8.12	35
	C ₄	0.78	4	0.83	4
	Σ	19.07	100	22.54	100
48 hrs. $\bar{x}_4$	C ₂	33.45	49	36.98	49
	C ₃	31.66	48	37.71	48
	C ₄	2.14	3	2.33	3
	Σ	67.25	100	77.02	100

$\bar{x}_4$: Each figure is the average of 4 determinations.

Table 16

Volatile Fatty Acids mM per Liter and Per Cent Molar (%M)
Contribution of Each Acid at Increasing Times of In Vitro
Cellulose Fermentation by Rumen Bacteria, With and Without
MHA or DL-Methionine--Trial #3

Fermt. Time	Acid	Control		MHA		DL-Methionine	
		mM/l	%M	mM/l	%M	mM/l	%M
0 hrs.	C ₂	5.99	70	5.91	77	5.84	75
	C ₃	1.74	21	1.14	15	1.24	16
	C ₄	0.86	9	0.59	8	0.67	9
	Σ	8.59	100	7.64	100	7.75	100
24 hrs.	C ₂	23.79	63	22.90	55	23.39	54
	C ₃	13.21	35	17.91	43	19.65	44
	C ₄	0.97	2	0.96	2	0.93	2
	Σ	37.97 ^{Aa}	100	41.77 ^b	100	43.97 ^B	100
48 hrs.	C ₂	47.59	49	53.45	49	57.41	49
	C ₃	46.61 ^A	49	55.21 ^B	49	55.95 ^B	49
	C ₄	2.02	2	2.32	2	2.49	2
	Σ	96.22 ^A	100	100.98 ^B	100	115.85 ^B	100

$\bar{x}_{2-5}$: Each figure is the average of 2, 4 or 5 determinations.

^{AB}Same letter or no superscript indicates no significant
(P<.01) difference within the same incubation time and
response

^{ab}Idem (P<.05)

of incubation in all treatments. The increase proceeded at a much faster rate for C_3 than for C_2 acids as indicated by their changing molar percentages. Data in Table 17 show that C_2 makes up about 70 per cent and C_3 about 17 per cent of the total VFA values at the beginning of incubation, but after 48 hours, C_2 and C_3 contributed equally or approximately 48 per cent each to the total VFA. The same trend is observed in Table 16.

This selective production of C_3 is not in accordance with what is observed usually in vivo or in vitro when rumen organisms ferment forages. The main difference in the present fermentation medium is the use of purified cellulose (Solka Floc) as substrate and the absence of protozoa.

The quantities of VFA's in Table 16 consistently show that values for MHA or DL-met medium are slightly higher (5 to 17 per cent) than in the control medium. The VFA values in Table 17 show that the C_3 fraction is higher ($P < .01$) for the MHA and DL-met media at 48 hours of fermentation of cellulose. The total VFA's values in Table 17 show that DL-met ($P < .01$) and MHA ($P < .05$) media had greater synthesis of VFA than the control medium at 24 hours.

At 48 hours of fermentation MHA and DL-met were higher ($P < .01$) in total VFA concentration than the control medium.

Values for individual as well as total VFA's for MHA and DL-met media are essentially the same.

The cellulose medium was assayed for lactic acid. This was done on the samples analyzed above for VFA's. The amount of lactate found for all treatment groups was less than 2 μg per ml of medium. Precise measurements were difficult since 1 $\mu\text{g}/\text{ml}$ was the lower resolution limit of the procedure used.

Barton et al. (1971) concluded that the optimum level of S in the ration DM should be between 0.14 and 0.17 per cent. The control medium in this study contained 0.11 per cent S from MgSO_4 in the 4 g of DM present per incubation bottle. Addition of Na_2SO_4 increased the S content to 0.15 per cent of the DM, resulting in a larger cellulose digestion and bacterial N production (sulfate treatment) which is in agreement with Barton's conclusion.

The need of sulfur for the utilization of non-protein nitrogen has been studied extensively in sheep (Bray and Hemsley, 1969); however, there is no agreement as to whether S containing amino acids or MHA have an advantage over sulfate as supplements for ruminants. Some researchers from Hunt et al. (1954) to Barton et al. (1971) are of the opinion that Na_2SO_4 is equal to methionine as an S source for in vitro cellulose digestion. On the other hand

Jacobson et al. (1967) believes that inorganic S is inefficiently utilized by ruminants and that methionine causes better utilization of feed in vivo. Polan et al. (1970a) have also shown a significant response in crude fiber digestion with the addition of MHA to rations of dairy cows.

The present experiments showed that when in vitro digestion of cellulose continued for 24 hours, methionine and MHA had a more pronounced effect on cellulose digestion and bacterial growth than sulfate. When fermentation continued for another 24 hours cellulose digestion as well as bacterial N were similar for MHA, methionine, and sulfate. This was due to an earlier cessation of growth (and even cellular hydrolysis) in the methionine- and MHA-containing bottles as a result of substrate dilution, catabolites built up, or both. This would not be the case in the rumen where microbes are turned over at intervals shorter than 24 hours and catabolites are rapidly removed.

The in vitro forage digestions of Hunt et al. (1954) and Barton et al. (1971) seem to have been determined after incubation times longer than 24 hours.

CHAPTER VI

IN VITRO FERMENTATIONS WITH STARCH AS SUBSTRATE-- EXPERIMENTAL PLAN, RESULTS, AND DISCUSSION

Effect of MHA, DL-Methionine, and Sodium Sulfate on Amylolytic Rumen Bacteria

An investigation was made with rumen bacteria to determine if they would benefit in a medium containing starch and urea from the addition of 10 μg of S per ml in the form of MHA, DL-met, or Na_2SO_4 . According to Kennedy et al. (1971) the S requirement of washed rumen bacteria for starch digestion in vitro is approximately 2 μg per ml of medium. The basal medium in this study already contained 28 μg of S per ml from MgSO_4 and should be adequate for starch digestion.

Two trials were conducted using three incubation bottles for each treatment group. Duplicate aliquots were taken from each bottle at 9 and 18 hours of incubation for analysis of starch digestion, bacterial N produced, and ammonia N in the medium.

The techniques and medium composition were the same as when glucose was the source of energy in previous trials, except that each incubation bottle contained initially about 3 g of purified corn starch as the only source of carbohydrate.

The inoculum was obtained from a steer fed a high corn ration. Residual starch was determined using the method described for dry matter (DM) and subtracting the weight of the bacterial N x 10 found in the DM. Percentage of starch digested was calculated using the average of 12 determinations of the starch value at the start of incubation.

Least-squares analysis of variance was applied combining the results of the two trials at each incubation time separately. Least-squares means for both experiments combined are presented in Table 17.

Duncan's multiple range tests for all combinations of the four treatments were made for each response at each of the two incubation periods independently. The data showed that starch digestion measured at 9 hours of incubation was improved by MHA ($P < .01$), DL-met ($P < .01$), and by Na_2SO_4 ($P < .05$). DL-met was more effective ($P < .05$) than sulfate but not more than MHA. Synthesized bacterial N at 9 hours was increased ($P < .01$) by the MHA and DL-met. The sulfate medium had less ($P < .01$) bacterial N than the

Table 17

Effect of MHA, DL-Methionine, and Na₂SO₄ on Starch Digestion, Bacterial Nitrogen (N), and Utilization of Ammonia N, Measured at 9 and 18 Hours of In Vitro Fermentation by Rumen Bacteria

Incubation Time/Hours	Control	MHA	DL-met	Na ₂ SO ₄
Starch digestion %				
9	2.60 ^{Aa}	5.75 ^{Bc}	7.70 ^{Bc}	5.23 ^{Ab}
18	41.8 ^A	47.2 ^B	41.5 ^A	43.3 ^A
Bacterial N mg/bottle ¹				
9	9.68 ^{Aa}	12.08 ^{Bc}	13.52 ^{Bd}	10.88 ^{Ab}
18	31.60 ^a	34.24 ^b	33.52 ^a	31.52 ^a
Ammonia N _i mg/bottle ¹				
9	24.9 ^A	23.0 ^B	22.8 ^B	25.2 ^A
18	20.2 ^A	22.1 ^{Aa}	26.6 ^B	20.9 ^{Ab}

Note: Each figure is the average of 12 determinations obtained from replicate trials 1 and 2.

^{AB} Same letters or no superscript indicate no significant ($P < .01$) difference within the same incubation time and response.

^{ab} Idem ($P < .05$).

¹ 160 ml of total medium.

MHA and DL-met media although sulfate medium was higher ($P < .05$) in bacterial N than that of the controls. DL-met stimulation was greater ($P < .05$) than that of MHA.

Ammonia consumption by 9 hours was accelerated ($P < .01$) by DL-met and MHA but not by Na_2SO_4 .

Starch digestion at 18 hours was increased ($P < .01$) by MHA only. Neither the DL-met nor Na_2SO_4 had any effect at this time.

At 18 hours MHA caused a greater ($P < .05$) bacterial N production, but DL-met and sulfate had no effect. In both trials bacterial N increase was proportional to percentage of starch digested.

Ammonia concentration in MHA and Na_2SO_4 media was not significantly different from control medium at 18 hours. The DL-met medium had higher ($P < .01$) values than any of the other treatments, probably due to deamination of the amino acid, especially in trial 1.

Overall these data would indicate that DL-met and MHA stimulated amylolytic rumen bacteria to grow faster during the first 9 hours of incubation, because the synthesis of the S-containing amino acids was a rate limiting factor in rumen bacterial development.

At 18 hours of fermentation only MHA continued to support increased metabolic activity. The failure of DL-met to maintain prolonged stimulation may be due in part to its

deamination or catabolism as indicated by Salisbury et al. (1971) who found that methanethiol production from L-methionine is much faster and reaches levels four times higher than from equivalent concentrations of MHA when incubated with rumen microorganisms.

The observations of the present study should help explain why Kennedy et al. (1971) found DL-met not to be superior to other sources of inorganic sulfur in stimulating starch digestion by rumen bacteria. The differences in vitro between MHA and DL-met may help explain why results, which for the most part have been negative with animals given diets supplemented with methionine, have been beneficial when concentrate rations were fed with MHA.

Conversion Efficiency of Substrate to Bacterial Cells

The conversion efficiency of substrate to protoplasmic rumen bacterial cells was calculated as the mg of bacterial N produced per mg of substrate that disappeared at different incubation times for each trial. This represents a measurement of efficiency relative to bacterial N rather than an absolute efficiency. Data are recorded in Table 18.

The average conversions were 3.39 per cent and 2.94 per cent for control and MHA treatments, respectively; and were

not significantly different by a t test analysis.

The large variability between trials may, in part, be due to the inadequacy of the methods used, but it may also reflect differences in efficiency at different times of incubation as the case when values close to the beginning and end of the logarithmic phase are considered within one experiment.

Lack of measurements on all products of substrate catabolism makes it difficult to explain such efficiency changes. Nevertheless, MHA appears to have no significant effect on the efficiency of substrate utilization for growth by rumen bacteria.

Table 18

Efficiency of Substrate to Bacterial N Conversion

<u>Data Obtained From</u>	<u>Incubation Time/Hours</u>	<u>Substrate</u>	<u>mg of bacterial N produced mg of substrate consumed</u>	
			<u>Control</u>	<u>MHA</u>
Table 2	6	Glucose	.011	.014
Figure 4,5	4.5	"	.017	.030
Figure 4,5	7	"	.028	.030
Table 6	7	"	.048	.032
Table 10	4.5	"	.066	.034
Table 14	24	Cellulose	.029	.026
Table 14	48	"	.012	.012
Table 18	9	Starch	.116	.065
Table 18	18	"	.023	.022

CHAPTER VII

IN VIVO EFFECT OF DIETARY MHA ON RUMEN MICROBIAL POPULATION AND LIPOPROTEINS OF SHEEP

Effect of Dietary MHA on Protozoa Counts and Bacterial Nitrogen in the Rumen of Sheep

Data on protozoa numbers and bacterial N in the rumen of MHA treated and control animals at 3, 12, and 18 days of MHA feeding are presented in Figures 11, 12, and 13.

Protozoa counts exhibited a large fluctuation in each rumen regardless of nutritional treatment. The numbers observed just prior to and 4 hours after feeding also varied greatly. Increases of either protozoa or bacteria were not consistently observed as a result of feeding. Total bacteria N taken as an estimate of the concentration of bacterial cells also presented such variability from day to day in each animal. Bacterial variability seemed to be inversely related to the protozoa numbers. Thus, bacterial N was low when protozoa numbers were high, and vice versa. This interdependence was obvious in the cases of sheep No. 16 and No. 17 as illustrated in Figures 12 and 13, respectively. The behavior was suggestive of a cyclic equilibrium characteristic of complex populations in which

predator species (protozoa) depend on their prey (bacteria) as source of food (Gutierrez, 1958). When conditions are favorable to the development of those preyed upon, the predator's development follows at a maximum rate; however, it starts to decline when the number of bacteria reaches a minimum.

The situation in the rumen is bound to be much more complex than this due to the large variety of different species of both protozoa and bacteria which proliferate at characteristic rates on different substrates.

Four sheep ate an average of 1100 g/day, but animals No. 16 and No. 17 consumed an average of 775 and 725 g/day, respectively. These two sheep were slow eaters and spaced their eating throughout the 24-hour period between feedings. Continuous eating is known to induce high numbers of protozoa (Moir and Somers, 1956). On the average these two animals exhibited about a 50 fold higher concentration of protozoa over that of the other four sheep. Nevertheless, protozoa concentration returned to normal on day 3 for animal 16 and day 12 for animal 17.

Larger concentrations of bacteria were associated with the decreased protozoa numbers on days 3 and 12.

These large microbiota changes took place regardless of treatment as demonstrated by sheep No. 16 which was on

MHA and sheep No. 17, a control animal.

Patton et al. (1970c) observed increased protozoa numbers due to MHA supplementation of animals fed an all ground grain ration. Such animals were under dietary conditions known to deplete protozoas. The sheep in the present study were fed a more conventional ration. The present experimental conditions were different and this may explain why our MHA supplementation did not have the same influence on protozoa. In the present study, fast or slow eating had a much more pronounced effect on protozoa numbers than MHA.

Some shortcomings in the techniques of the present study make it difficult to draw quantitative inferences. The total volume of the rumen was not measured; therefore, the day-to-day or before-and-after feeding variation may to some extent be a consequence of differences in dilution. The present values for protozoa numbers and bacteria protein fall within the wide range found in the literature. The wide range in the literature may also be attributable in part to differences in dilution. In addition, the values for ruminal bacterial nitrogen for the slow eating animals No. 16 and No. 17 may not reflect the bacterial concentration to the degree that the other animals did. There was no significant difference between N content of rumen fluid before and after eating and the protozoa-bacteria ratio

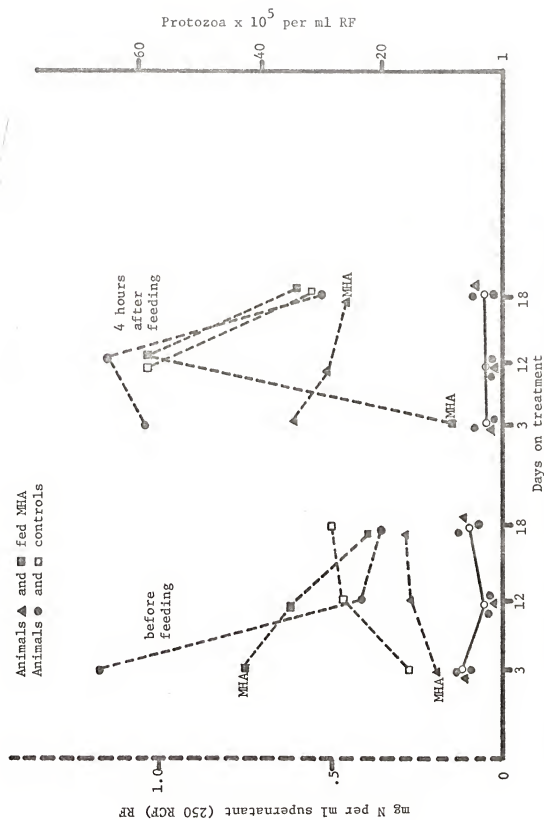


FIGURE 11. EFFECT OF DIETARY MHA ON PROTOZOA NUMBERS AND BACTERIAL N IN RUMEN FLUID (RF) DETERMINED JUST PRIOR TO AND FOUR HOURS AFTER FEEDING; ON DAYS 3, 12, AND 18 AFTER STARTING MHA SUPPLEMENTATION

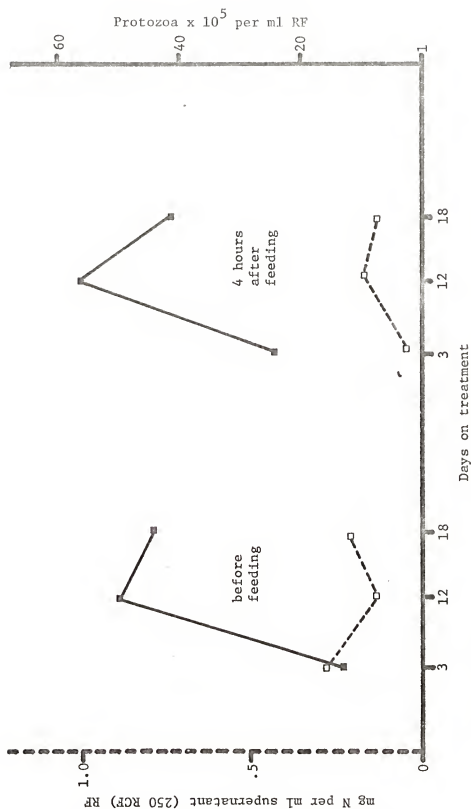


FIGURE 12. EFFECT OF MHA ON PROTOZOA NUMBERS AND BACTERIAL N IN RUMEN FLUID (RF) OF ANIMAL NO. 16, JUST PRIOR TO AND FOUR HOURS AFTER FEEDING, ON DAYS 3, 12, AND 18 AFTER STARTING MHA SUPPLEMENTATION

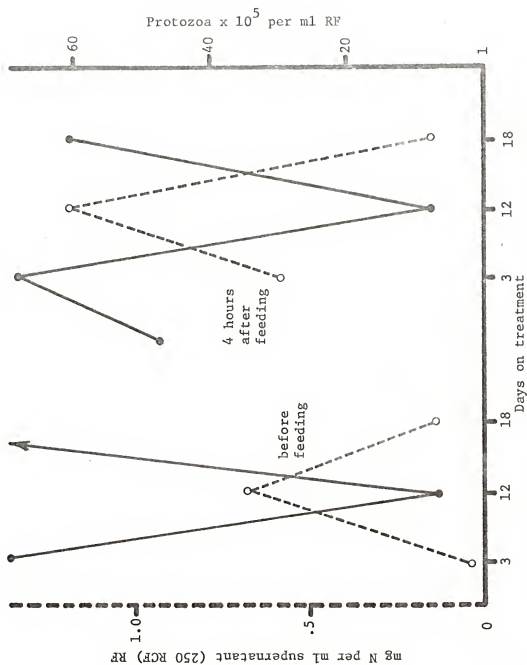


FIGURE 13. PROTOZOA NUMBERS AND BACTERIAL N IN RUMEN FLUID (RF) OF CONTROL ANIMAL NO. 17, ON DAYS 3, 12 AND 18 AFTER STARTING MHA SUPPLEMENTATION

remained the same after eating for either slow or fast eaters. This indicates that if there were an error made in equating ruminal nitrogen with bacterial mass, it was not due to shifting of protozoa-bacteria ratio.

Lipoprotein Concentration

Weekly and general averages of HDLP for MHA and control groups are presented in Table 19. The MHA group had a higher ($P < .05$) average HDLP throughout the experimental period. The weekly averages had a decrease in both treatment groups of about 500 $\mu\text{g/ml}$ from week to week. No significant differences were observed for the LDLP concentration of the two groups (Table 20). The weekly values in the MHA group showed a gradual increase in LDLP while the control had a gradual decrease in LDLP weekly averages.

The HDLP/LDLP ratio (Table 20) gradually decreased from 7.70 to 5.47 to 3.53 from the first to the second and third week for the MHA group. Such a trend did not occur in the control group.

The VLDL fractions were found to be undetectable by our experimental procedure.

These observations indicate that lipoproteins are influenced by MHA but that other factors are also present. Obviously, more studies are needed to establish what other

Table 19

Effect of MHA on High Density Lipoproteins (HDLP) and Low Density Lipoproteins (LDLP) in Serum of Sheep During Three Weeks of Feeding

weekly averages of HDLP mg/ml				
<u>Days of Treatment</u>	<u>3</u>	<u>12</u>	<u>18</u>	<u>Total $\pm$ S.E.</u>
MHA	2703	2153	1660	2172 $\pm$ 205
Control	2333	1750	1266	1789 $\pm$ 240
weekly averages of LDLP mg/ml				
MHA	351	393	470	405 $\pm$ 37
Control	555	493	286	431 $\pm$ 58

Table 20

Ratios of HDLP/LDLP From Values Reported in Table 19

<u>Days on Treatment</u>	<u>3</u>	<u>12</u>	<u>18</u>
MHA	7.70	5.47	3.53
Control	4.20	3.54	4.42

factors are involved. It would give more confidence in the test if the control sheep were unchanging in lipoprotein concentration during the observation period.

The present results are comparable to the limited data reported on other ruminant lipoproteins (Fried et al., 1968) in that the HDLP concentration is from 3 to 5 fold as abundant as the LDLP and that the VLDL fraction contributes very little to the total lipoproteins.

Amino Acid Composition of Lipoproteins

No significant differences in amino acid composition were found between HDLP and LDLP lipoproteins of the MHA treated and control group as averaged and presented in Table 21. The level of methionine in the HDLP of both control and MHA groups were undetectable. Neither MHA supplementation, eating habits, or lipoprotein concentration had any detectable effect on the percentages of amino acids.

The amino acid compositions observed here suggest that they are not an important factor in the differentiation of the HDLP and LDLP fractions, unless the absence of methionine at a relatively small concentration in the case of the HDLP is a dominating factor.

Table 21

Per Cent Amino Acids Composition of LDLP and HDLP
in Serum of Sheep¹

	LDLP		HDLP	
	<u>% $\bar{x}$</u>	<u>S.D.</u>	<u>% $\bar{x}$</u>	<u>S.D.</u>
Lysine	6.95	1.1	8.48	.64
Histidine	2.36	.33	1.70	.15
Arginine	3.31	.14	4.81	.66
Aspartic Acid	10.75	.36	9.43	.84
Threonine	5.99	.45	2.91	.55
Serine	6.69	.47	3.92	.41
Glutamic Acid	13.52	.68	20.96	.73
Proline	4.29	.38	3.87	.34
Glycine	5.95	.37	5.10	.42
Alanine	6.88	.40	11.31	1.93
Valine	6.50	.11	5.67	.98
Methionine	.34	.11	0	0
Isoleucine	4.61	.59	1.37	.14
Leucine	11.64	1.52	14.02	1.05
Tyrosine	3.01	.35	2.20	.22

Table 21 (continued)

	LDLP		HDLP	
	<u>% $\bar{x}$</u>	<u>S.D.</u>	<u>% $\bar{x}$</u>	<u>S.D.</u>
Phenylalanine	4.79	.29	2.69	.22
Gal-NH ₂	.51	.24	.38	.16
Glu-NH ₂	1.47	.32	.84	.23
Cysteic Acid	.57	.14	.34	.10

¹Each figure is the average of six determinations of which three were from animals receiving MHA and three from control animals. Each of the three pairs of values was obtained at a one-week interval after MHA feeding was initiated.

GENERAL DISCUSSION

(Most amino acids had a slight stimulation on growth of cells utilizing glucose, which shows that the energy spared in the synthesis of an amino acid can be diverted to other metabolic activities of growth.) Only the sulfur-containing amino acids caused growth to proceed at twice the rate of unsupplemented bacteria. However, the energy necessary for synthesis of the sulfur-containing amino acids does not appear to be an appreciable quantity judging from the unmodified efficiency of substrate to cellular mass conversion. (The growth stimulation indicates that the change of inorganic sulfur to sulfur-containing amino acids is a slow process that limits the rate of protein synthesis and, therefore, rumen bacterial growth.)

Data on substrate digestion indicate that an inorganic sulfur level of 28 μ g/ml present in the control media from $MgSO_4$, was adequate for starch digestion, therefore addition of sodium sulfate had no improving effect. Kennedy et al. (1971) reported that the requirement for starch digestion was about 2 μ g S per ml. The same concentration of sulfur

in the basal medium (.11 per cent of the DM) was insufficient for cellulose digestion. This is in agreement with Barton et al. (1971) who reported a sulfur requirement of about .15 per cent of the DM; this level was attained by addition of Na_2SO_4 to the basal medium and resulted in an improvement of the cellulose digested over a 48 hour period.

Trials with glucose at concentrations equivalent to those of starch and cellulose showed that all of the glucose is digested after about 8 hours regardless of the sulfur level. However, any of the S-containing amino acids increased the glucose digestion rate and concomitantly accelerated bacterial growth. Clearly if the only response measured was glucose digestion after 8 hours of incubation, no differences would be seen between organic and inorganic sulfur.

The same precaution should be exerted when 48 hours of cellulose digestion is measured to evaluate sulfur sources. Neither cellulose digestion nor bacterial N were significantly different at this time. This was a consequence of bacterial degradation caused by depletion of substrate and/or accumulation of catabolic products as evidenced by the higher cellulose digestion and bacterial N generally found at 24 hours with MHA and Methionine but not with sulfate supplementation. In trials with starch as the substrate,

incubation was not prolonged beyond the end of the logarithmic growth phase, but at 18 hours only MHA continued to support accelerated bacterial growth.

It is felt, in general, that the importance of providing MHA to rumen bacteria would be to increase the rate of substrate digestion and the amount of bacterial protein synthesized during the time that bacterial cells remain in the rumen, which is presumably shorter than 24 hours.

The applicability to practical feeding of observations made with rumen bacteria cultured in glucose as substrate is questionable. The present data indicated a shift in the bacterial species based on the changes in the amino acid composition of the bacterial protein developed in glucose as well as the presence of lactic acid as the main catabolic product of glucose which might be indicative of lactobacilli development. If such were the case, the cellulolytic properties of such population would have been almost eliminated instead of reduced to 50 per cent as was the case. Furthermore, the flourishing of lactobacilli is usually brought about by a lowering in the rumen pH to about 5. The incubation medium used here was buffered at pH 6.9 and corrected whenever the pH dropped 0.5 units by adding drops of a sodium carbonate solution. More in accordance with these conditions is to suppose that the lactic acid producers that developed

in glucose could be Cillobacterium cellulosolvens and/or Streptococcus bovis a cellulolytic and amylolytic specie respectively both lactic acid producers and normal dwellers of the rumen.

Since cause of lactic acid production in the rumen is not a thoroughly understood phenomenon, it may be hypothesized that increase demand for energy such as accelerated growth of these species in glucose and in the presence of MHA requires a rapid reoxidation of DPNH which can be accomplished by the reduction of pyruvate to lactate.

Methionine acts as a glucogenic amino acid in animal tissue. However, rumen bacteria which have depleted all of their available glucose substrate before reaching the ecological limit of the fermentor were unable to utilize MHA as a substrate or an energy source for growth. Nevertheless, MHA and methionine were catabolized by the bacteria as evidenced by a decrease of approximately 20 mg in about 7 hours of incubation. Based on the per cent of methionine found in the bacterial protein and the weight of bacterial cells produced, this would mean that about 1 mg of methionine was incorporated into bacterial cells, and 19 mg was converted to catabolic products. Zikakis and Salisbury (1969) showed that S-containing amino acids are converted to volatile sulfurous rumen gases, but no attempt was made at quantitating

the proportion of the amino acid which is lost as gases; apparently, it depends on the concentration to the point that if there is no free methionine in the presence of these sulfurous compounds, the equilibrium of the reactions would be displaced in the direction of the synthesis of the amino acid.

Data of this study are in agreement with Emery (1971) who reported that MHA and methionine disappears from the rumen at about the same rate, but contrast with Bishop's (1970) who reported a long stability of MHA compared to that of methionine in rumen fluid. Our data also indicated that MHA in excess of the bacterial requirement is less effective in promoting growth. All these data may suggest that the form of administration can be an important factor in the utilization of MHA by the rumen bacteria; if a single dose is given once a day, the loss of the compound may be large while if sustained low concentrations are available, the bacterial utilization may be complete and the protoplasmic protein production highly improved. Obviously, the question of whether MHA exerts its main effect systemically on the ruminant or whether it is mediated through the rumen microbes is a crucial one. Up to now, the rumen bypass and systemic effect theory has been accepted, thus, the general practice

has been the single dose administration. The author feels that this practice may account for a good number of negative results.

Methionine is known to be converted to cysteine in animal tissue, but the reverse has never been shown. Rumen bacteria seem to have the capacity to convert any of the S-containing amino acids into the others very rapidly, as demonstrated by the growth stimulation caused by any of the S-amino acids or MHA.

CONCLUSIONS

Incubating rumen bacteria in a medium that contained urea as the only source of nitrogen and 28 µg of sulfur per ml from MgSO_4 , the following conclusions were made:

1. MHA added to a medium containing glucose causes bacterial growth rate to double as measured by bacterial dry matter, crude protein synthesis, and glucose utilization.

2. MHA added to a medium containing cellulose causes an increase in cellulose digestion of about 10 per cent by 24 hours and about 20 per cent by 48 hours of incubation. Similar results are observed for bacterial crude protein and utilization of ammonia.

3. MHA added to a medium containing starch causes an increase in starch digestion of approximately 5 per cent by 18 hours or incubation with comparable increases in bacterial protein synthesis and ammonia utilization.

4. DL-methionine is equivalent to MHA in increasing rumen bacteria utilization of glucose or cellulose, but only MHA has a prolonged effect on starch digestion presumably because DL-methionine is rapidly destroyed by amylolytic bacteria.

5. Sodium sulfate added to a medium containing cellulose increases degradation of cellulose and bacterial growth at 48 hours of fermentation but not at 24 hours. This indicates that the basal medium is deficient in S and synthesis of methionine from sulfate is a growth rate limiting process for cellulolytic bacteria.

6. Culturing rumen bacteria in a medium containing glucose seems a convenient way to evaluate growth factors in vitro. By this method, the following properties of MHA are disclosed:

MHA is not utilized as a readily available source of energy by rumen bacteria;

Cysteine, cystine, and methionine are essentially equivalent to MHA in stimulation of glucose utilization and protein synthesis by rumen bacteria in a medium containing glucose. Fifteen other amino acids or inorganic sources of sulfur do not have such effect on rumen bacteria;

MHA does not affect the production of total VFA's in a medium containing glucose, but it increases the proportion of propionic acid to acetic acid, and results in high concentrations of lactic acid. The same shift in VFA is observed when rumen bacteria utilize pulverized purified cellulose.

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BIOGRAPHICAL SKETCH

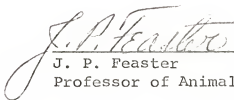
Luis Arturo Gil P. was born on August 20, 1935, in Bogota, Colombia. He graduated from Colegio Americano (high school) in December, 1955. He served in the Colombian Army from 1956 to 1957. In December, 1961, he completed his college work in the National University of Colombia and received a Bachelor of Science degree in Pharmaceutical Chemistry in October, 1963. During the period of January, 1962, to August, 1964, he worked as a chemistry instructor in the School of Medicine of the National University of Colombia. From September, 1964, to September, 1966, he attended the University of California at San Francisco and Berkeley and graduated with a Master of Science degree in Nutritional Biochemistry in September, 1966. Since that time until July, 1969, he worked as an assistant professor of Biochemistry in the school of Chemistry at the National University of Colombia. In September, 1969, he entered the University of Florida where he is a candidate for the degree of Doctor of Philosophy in Animal Sciences, at the present time.

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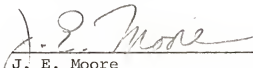
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Professor of Animal Science

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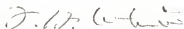
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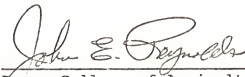
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March, 1972


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